1-2010

Carnosine content and antioxidant activity from poultry co-products, protein meal and stressed poultry tissues

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CARNOSINE CONTENT AND ANTIOXIDANT ACTIVITY FROM POULTRY CO-PRODUCTS, PROTEIN MEAL AND STRESSED POULTRY TISSUES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Food Technology

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by
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May 2011

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ABSTRACT

Four separate studies were conducted to examine carnosine levels and associated antioxidant activity in poultry co-products, in rendered poultry protein meal, in tissues from stressed or non-stressed chickens. In the first study, carnosine was extracted from poultry co-products (head, liver, lungs, tail, gizzard, brain and heart). Liver contained the highest (102.29 mg/gm) level, while brain contained the lowest level of carnosine (0.95 mg/gm) ($p \leq 0.05$). Except brain, all tissue ultrafiltrates (20.87-39.57%) and reconstituted dry powders (5.66- 14.47%) showed thiobarbituric reactive acid species (TBARS) inhibition. Head ultrafiltrate and reconstituted dry powder showed maximum while gizzard showed the minimum metal chelating activity ($p \leq 0.05$). Free radical scavenging activity of ultrafiltrate of all tissues samples ranged from 25.1 to 79.4% while this activity was higher (29.8 to 84.1%) in the reconstituted dry powder of all tissue samples. Oxygen radical absorbing capacity (ORAC) values were highest in liver ultrafiltrate and lowest in heart ($p \leq 0.05$). Results indicated that carnosine was present in all the tissue samples investigated and their ultrafiltrates as well as dry powders of tissue samples possess antioxidant properties.

In the second study examining poultry protein meal, carnosine content of sample-G was almost 2.6 times higher than sample–A. TBARS inhibition by sample-G was 15.9% while Sample-A did not exhibit any TBARS inhibition. Metal chelating activity and free radical scavenging activities of sample-A and sample-G did not differ. ORAC values (µM Trolox Equivalents /gm of dry sample) of sample-A (84.4) were greater than sample-G (68.4) ($p \leq 0.05$).

The third study determined carnosine levels in different tissues of broilers under stress versus non-stress conditions. Corticosterone levels of stressed broilers (24.36 ng/ml) was 10 fold higher ($p=0.002$) than non-stressed broilers (2.28 ng/ml). There was significant increase in carnosine content in breast tissue of stressed birds (17.39 mg/gm), and was 10 times ($p=0.005$) more than non-stressed birds (1.85 mg/gm). Carnosine content in thigh of stressed birds (21.25 mg/gm) was approximately 2 fold higher ($p=0.001$) than non-stressed birds (11.10 mg/gm). Carnosine content in brain of stressed birds did not differ ($p=0.82$) from that in non-stressed birds. Results indicated that carnosine may play a significant role in muscles during short term stress.

In fourth study, it was determined that TBARS inhibition and metal chelating activity of carnosine was due to the imidazole ring present in the histidine while free radical scavenging activity of carnosine was attributed to histidine amino acid.

Overall, conclusions were drawn that poultry byproducts, poultry protein meal contains carnosine and exhibited antioxidant properties. These antioxidant properties were due to carnosine's unique structure. Lastly, stress increases the carnosine levels in breast and thigh tissues of broilers.

DEDICATION

I dedicate this work to my parents and family, who always supported me to make this a success.

ACKNOWLEDGEMENTS

First of all I would like to thank God for giving me strength and wisdom to complete my research thesis. I would like to thank Dr. Paul Dawson as my advisor and mentor without his innovative ideas and intellectual, financial and moral support this research would not have been accomplished.

I would like to express my sincere thanks to Dr. J.K. Northcutt for being a guide and for her intellectual and experimental assistance, friendly behavior and conducive support throughout this research.

I would like to thank to Dr. W.C.Bridges for helping me in solving tough problems of statistics and analyzing the data and for being there with an answer to all my statistics questions.

Dr Thomas Scott, besides being busy on dean's duty was willing to give his valuable time as my committee member and as teacher for answering cell and immunology questions and guiding me through possible experimental procedures, many thanks for your help and support.

I would to express my sincere thanks to Dr. I. Han for being there as a friend and as a guide to provide me with moral and intellectual support during rough times.

From the core of my heart, I would like to thank my parents, S. Harbans Singh Manhiani and Surinder Kaur Manhiani without them this dream would not have come true. My brothers Sukhwinder Singh Manhiani, Rajwinder Singh Manhiani, Maninder Singh Manhiani and my sister-in-laws Darshana Manhiani and Marlina Manhiani for their emotional and moral support throughout this long journey. My loving nephews,

Rupinder, Sammy, Molu and Golu for their love and affection and a cute question: What is your class teacher's name?

I would like to recognize my wife, Jaspreet for her encouragement and moral support. For her helpful discussion about the experimental work and as intellectual guide to ask very difficult and critical questions about my research and then support or disagree about the answer I gave.

I would like to thank Jason Raines for being as my room-mate and a person to discuss my experimental problems.

I thank all my friends and lab mates especially Divija, Chaitali for their help and support in my sample preparations.

I would like to thank Dr. Pometto and Dr. Haley-Zitin for letting me use the HPLC.

And lastly, from the bottom of my heart, I would also like to thank the entire Food Science and Human Nutrition Department , and Department of Animal and Veterniary Sciences, all the faculty and staff for their direct or indirect help in this research and for their support throughout this program.

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CHAPTER ONE

INTRODUCTION

Oxidation in foods is a major concern in food deterioration affecting lipids, proteins and carbohydrates. However, oxidation of lipids is the main cause of oxidative deterioration of food leading to loss of sensory and nutritional quality. Oxidation may also form toxic compounds which could pose potential health concerns, such as atherosclerosis, cytotoxicity, and carcinogenesis. Therefore, preventing oxidation of food is critical to increasing shelf life and maintaining the quality of foods.

Oxidation of lipids is a chain reaction which can be depicted in three steps; initiation, propagation and termination. Antioxidants are substances added to food to retard the rate of the oxidation reaction primarily during initiation and to a lesser extent propagation. While antioxidants can inhibit oxidation in several ways, "true antioxidants" act by inhibiting the chain reaction:

$$\mathbf{ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot}}$$

Food antioxidants can be categorized as either natural or synthetic. Tocopherols, ascorbic acid, polyphenols and quercetin are the examples of natural antioxidants, while butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG) and tert butyl hydroquinone (TBHQ) are synthetic antioxidants. Synthetic antioxidants are strictly regulated by the Food and Drug Administration (FDA), due to lingering concerns about their toxicity above certain limits.

Due to the positive perception of natural foods and positive health implications for antioxidants, numerous research studies are being conducted to identify natural

antioxidants from different sources and to minimize the use of synthetic antioxidants and to enhance the nutritional quality of food. There is interest in carnosine as a natural antioxidant for the food industry, especially since there are no reports of toxic effects of carnosine since its derivatives and excess of dietary carnosine is excreted in urine. Since its discovery in 1900 by Guelwitsch from beef extract, carnosine has been extracted from a variety of animal tissues. Researchers have also sought to better understand the significance of carnosine in the human body. Carnosine has been identified as a neurotransmitter, buffer in the skeletal muscle, aid in muscle contraction, regulator of cardiac muscle proteins, anti-ageing factor and a potent skeletal muscle antioxidant. Carnosine exhibits multiple antioxidant properties such as metal chelation, reactive oxygen scavenging, free radical scavenging and peroxide decomposition. With multiple modes of antioxidant activity in foods and in the body there is keen interest in the scientific community in carnosine.

In the US, 100 million hogs, 35 million cattle and 8.7 billion chickens are processed annually. Foury nine percent live weight of cattle, 44% live weight of hogs, 37% live weight of poultry and 57% live weight of the fish products are not used for human consumption. The unprocessed or underutilized products of the food or animal industry could pose a serious hazard to the environment, humans and animals, if not handled properly. The rendering industry processes these inedible products producing valuable by-products. Presently, in the United States (US), there are approximately 300 rendering plants processing about 100 million pounds of meat industry waste every day, resulting in total production of 54 billion pounds processed co-products annually.

In 2009, 8.7 billion chickens were slaughtered in the US resulting in production of 1.16 million metric tons of poultry byproduct meal, 0.6 million metric tons of poultry fat and 0.5 million metric tons of feather meal (Swisher, 2009). At present, most poultry by-products are being utilized by the rendering industry to produce poultry by-product meal and fat. Carnosine has already been extracted from low value products such as mechanically deboned pork and from isolated muscle protein waste material.

Therefore, the objectives of the present research were:

1. To determine the carnosine content in poultry by-products and to measure its antioxidant properties
2. To determine the carnosine content in poultry protein meal and to measure its antioxidant properties.
3. To compare carnosine antioxidant activity with its constituent amino acids, β-alanine and L-histidine as well as with imidazole.
4. To compare carnosine levels recovered from breast, thigh and brain tissues of broilers previously exposed to short term or non-stress conditions.

One potential of this research would be the recovery of carnosine from poultry co-products prior to rendering. If 3.4 billion pounds poultry by-products are considered as raw material for carnosine recovery, without considering organic nature and purity, it can be estimated that approximately $3.07 million income could be generated by extracting carnosine from these poultry by-products. Therefore, the overall objective was to extract

carnosine using a single step water extraction procedure, with the ultimate goal of potentially increasing the revenue of the poultry processing and/or rendering industry.

Carnosine has numerous medicinal and therapeutic applications as well as antioxidant properties in the body. It has been reported that oral intake of carnosine improves high intensity exercise performance and endurance, facilitates wound healing, inhibits inflammation and has anti-ulcer effects due to membrane protection activity. Commercially, this dipeptide is being used as a therapeutic drug and in making anti-ageing skin lotions. However our primary aim was its application in animal food and as a therapeutic agent for animals.

CHAPTER TWO

LITRATURE REVIEW

2.1. Introduction

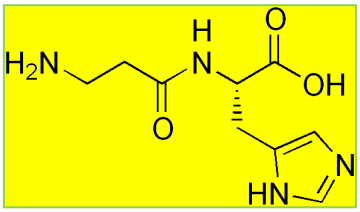
More than a century ago, in 1900, a crystalline substance called carnosine was isolated from beef muscle extract by Guelwitsch (Guelwitsch 1900, 1906 and 1911). From systematic analysis, it was found that carnosine is a water soluble dipeptide composed of β-alanine and histidine. Out of these two amino acids, histidine is an essential amino acid while β-alanine is a non-essential amino acid synthesized in liver as a final product of uracil and thymine degradation (Matthews and Traut, 1987).

Since 1900, voluminous research has been conducted studying the sources of carnosine, its physiological roles and its biochemical importance as well as studies into carnosine's therapeutic and medicinal properties.

2.2. Properties of Carnosine \

Carnosine is soluble in water (one part to 3.2 parts of water at 25°C) while insoluble in alcohol and most organic solvents, but can be solubilized in some organic solvents to a certain extent by first dissolving in an aqueous medium and then in organic solvents. Carnosine is basic in nature and possesses buffering capacity at pH 5.4 to 6.0 (Vigneaud and Behrens, 1939; Quinn et al., 1992).

Figure 2.1: Carnosine Structure



The structure of carnosine is closely tied to its functionality, according to a study conducted with chicks by Tsuneyoshi et al (2007), Carnosine (β-alanine-L-histidine) induced hyperactivity in chicks while the reverse structure of carnosine (L-histidine –β-alanine) induced the reverse effect, producing hypnotic and sedative effects in chicks. The reverse structure of carnosine showed similar results to β-alanine alone. Moreover, carnosine possesses antioxidant properties due to the imidazole moiety in its structure (Kohen et al., 1988; Aruoma et al., 1989; Hartman et al., 1990; Quinn et al., 1992; Vigneaud and Behrens, 1939; Boldyrev et al., 1993; Boldyrev et al., 1997).

2.3. Biosynthesis of Carnosine

Carnosine is synthesized from β-alanine and histidine by carnosine synthetase [EC 6.3.2.11] (Horinishi et al., 1978). Kalyankar and Meister (1959) found that Mg^{2+} and ATP are required for carnosine synthesis. β-alanine, ATP, and carnosine synthetase in the presence of Mg^{2+} form a β-alanine-adenylate complex which combines with L-histidine to produce carnosine.

William and Winnick (1954) studied the physiological site of carnosine and anserine formation in hatched chicks. They reported that skeletal muscle is the primary tissue for biosynthesis of both dipeptides while liver does not play any role in carnosine and anserine formation. Carnosine is methylated to a greater extent than histidine, and in-vivo as well as in vitro experiments, showed that methylhistidine condenses more readily with β-alanine than histidine.

Biosynthesis of carnosine is restricted to muscle cells, oligodendrocytes, and ensheathing cells of the olfactory bulb and increases during differentiation of these cells. Astrocytes, in contrast, do not synthesize carnosine but are equipped with a dipeptide transporter by which carnosine is taken up very efficiently (Bakardjiev and Bauer, 2000). A study conducted on rats showed that carnosine is synthesized in gastrocnemius muscle and not in liver (Tamaki et al., 1980). The half life of carnosine is 29 days and the rate of carnosine biosynthesis was 0.321 µmol/wet tissue (gm)/day. A similar study by David Fisher and his colleagues in 1977 on muscle and brain of developing embryos of chicks (Gallus gallus) found that carnosine levels in muscle of chick embryos were undetectable until 14 days while at 15 days carnosine levels were detectable < 3µmoles/100 gm and increased to 22.5 µmoles/100 gm a day after hatching. In contrast, carnosine levels were less than 3 µmoles/100 gm in brain of a day old chick, indicating that carnosine first forms in the muscle and then in the brain in chicks (Fisher et al., 1977).

2.4. Metabolism of Carnosine

Carnosine can be taken orally and can be synthesized in the body. Carnosine is absorbed maximally in the jejunum in rats which corresponds to the site for active D-glucose uptake and for uptake of most amino acids. Carnosine uptake is Na^+ dependent. In intact mucosal cells, Na^+ gradients stimulate peptide transport by producing a proton gradient via Na^+- H^+ exchange (Ferraris et al., 1988). Excess of carnosine is excreted in urine (Perry et al., 1967) and there are no reports of toxic effects of carnosine and its derivatives (Quinn et al., 1992; Sato et al., 2008) .

Carnosine is catabolised into β-alanine and L-histidine by carnosinase (Pegova et al., 2000). In humans, carnosine is hydrolyzed by two isozymes: tissue (cystolic) carnosinases [E.C.3.4.13.3] and serum carnosinase [E.C.3.4.13.20] (Scriver and Gibson., 1995). Guitto et al (2005) stated the carnosinase is not one enzyme but a group of intracellular and extracellular enzymes belonging to the large family of metalloproteases which have specific and cystolic non-specific roles. In 2003, Tuefel and his colleagues reported discovery of two novel genes CN1 and CN2 which are responsible for coding of metallopeptidases of the M20 family and demonstrated that CN1 corresponds to the secreted human carnosinases while CN2 is the cystolic non-specific dipeptidase (Teufel et al., 2003).

2.5. Regulation of Carnosine Levels

Physiological levels of carnosine are regulated by the activity of carnosine synthetase (regulates biosynthesis) and carnosinase (regulates metabolism), which are both present in brain (Quinn et al., 1992; Horinishi et al., 1978). Seely and Marshal (1982) determined the inhibition of carnosine synthetase by β-alanine analogues {3-aminopropane sulfonic acid (APS), 5-aminovaleric acid (5-AV), 2-aminoethyl phosphonic acid, o-phosphoethanolamine, nipecotic acid and aminooxyacetic acid} in rat and chick muscle. Upon isolation of these compounds after injection, they found that APS was most effective in inhibiting carnosine synthetase in both rat and chick by competitive inhibition while other analogues showed less inhibitory activity. Synthesis of carnosine could also be inhibited by 1- methyl histidine and 3- methyl histidine (Horinishi et al., 1978).

Carnosine can be metabolized into different derivatives such as anserine and ophidine by methylation and to acetyl-carnosine by acetylation. The pathways of metabolism of carnosine have been suggested by various scientists (Begum et al., 2005; Chan and Decker, 1994; William and Winnick, 1954; Kalyankar and Meister, 1959). The general pathway of carnosine metabolism is as follows:

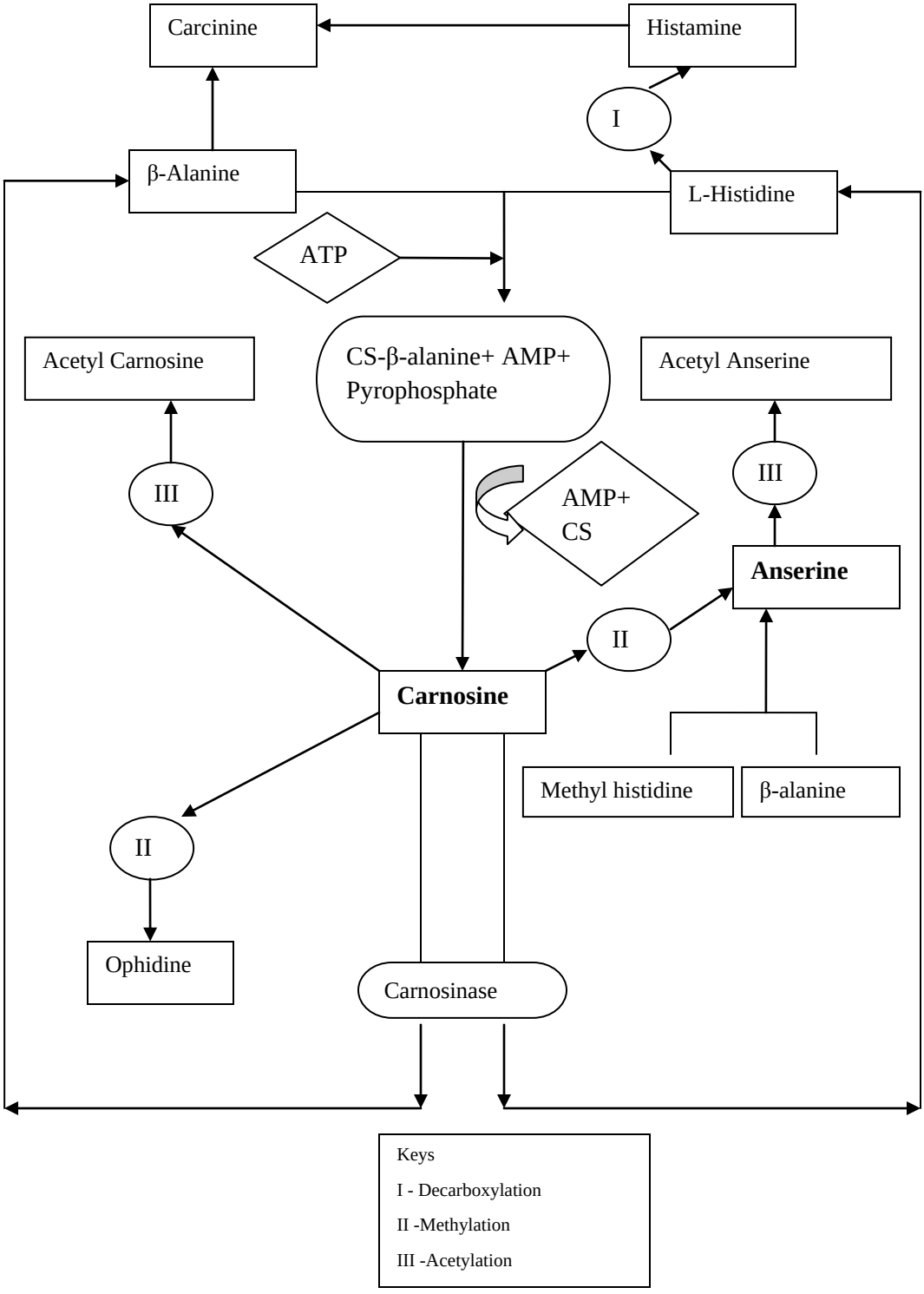


Figure 2.2: Pathways of Carnosine Biosynthesis and Metabolism.

Keyword: Carnosine Synthetase AMPEC 6.3.2.11= CS

2.6. Physiological Functions of Carnosine

Carnosine is responsible for a variety of physiological activities. Review articles have explained various physiological roles of carnosine in the contraction of muscles (Begum et al., 2005), as an antioxidant (Chan and Decker., 1994; Guiotto et al., 2005; Marchis et al., 2000) and as an anti-ageing compound (Stvolinsky and Dobrota, 2000). Protein carbonylation and accumulation of modified polypeptides causes age associated phenomenon among cells and tissues and thus could cause atherosclerosis, muscular dystrophy and neuro degenerative diseases such as Alzheimer's disease, Parkinson's disease. Various pathways of protein carbonylation have been suggested by Aldini et al (2005). Carnosine acts as anti-ageing compound by quenching or detoxifying cytotoxic carbonyls such as 4-hydroxy-tran-2, 3,-nonenal (HNR), acrolein (ACR) and malondialdehyde (advanced lipid oxidation end products) (Aldini et al., 2005). Table 2.1 provides a summary of various physiological functions of carnosine along with a mode /mechanism of action.

Table 2.1: Physiological Functions of Carnosine

S. No.	Physiological Function	Mode/ Mechanism of Action	Reference
1.	Muscle Contraction	Facilitates muscle contraction. Carnosine activates myosine ATPase.	(Severin et al., 1963; Avena and Bowen, 1969)
2.	Potent Buffer in Muscle	pH buffering is main function of carnosine, in muscle; carnosine and anserine contribute 40% of the buffering in the physiological range (pH 6.5 to 7.5). Supplementation of carnosine causes increased muscle carnosine concentrations which lead to increased intramuscular hydrogen ion (H^+) buffering capacity.	(Skulachev, 2000; Davey, 1960; Smith, 1938) (Abe, 2000; Dunnett and Harris, 1999)
3.	Neuromodulator	Transmission of nerve impulse.	(Severin et al., 1963; Trombley et al., 2000)
4.	Putative Neurotransmitter in brain	Acts as neurotransmitter.	(Trombley et al., 2000 ; Tomonaga et al., 2004; Tomonaga et al., 2005; Bonfanti et al., 1999)
5.	Nervous system	Carnosine protects neurons against oxidative stress produced due to ROS (Reactive Oxygen Species). Under oxidative stress on neurons, carnosine and anserine regulate cell stability and participate in the selection of preferred pathway for neuronal cell death (necrosis or apoptosis).	(Boldyrev et al., 1999) (Boldyrev, 2000)

6.	Behavioral attributes	Protects against amydaloid kindled seizures in rats.	(Jin et al., 2005)
7.	Potential treatment for Alzheimer's Disease (AD)	AGE (Advance Glycation End Products) and RAGE (Receptors for Advance Glycation End products) accumulate in amyloid plaques in AD. Carnosine attenuates the formation of AGE and thus can be a potential treatment for AD.	(Reddy et al., 2005)
8.	Anti-ageing properties	Carnosine acts as a potential anti-senescence drug, improves external appearance, and physiological functions in experimental mice. Carnosine reacts with protein carbonyls to form protein carbonyl carnosine adducts called "carnosinylated" proteins; thus produces anti-ageing and rejuvenating effects. Carnosine attenuates the formation of age presumably by sequestering reactive 1, 2- dicarbonyl compounds.	(Gallant et al., 2000) (Hipkiss and Brownson, 2000) (Reddy et al., 2005; Boldyrev et al., 1999)
9.	Minimizing DNA Damage	Helps in minimizing DNA damage by sequestering ROS (reactive oxygen species; H_2O_2, superoxide, hydroxyl radicals, HOCl) and RNS (reactive nitrogen species; Peroxynitrite, HNE: trans-4-hyroxy-2-nonenal).	(Reddy et al., 2005)
10.	Immune system	Helps in building immunity. Cofactor in defensive action of cortisones.	(Knight, 2000) (Nagai, 1971)

11.	Cardiovascular Effect	Carnosine acts as a modulator of calcium regulated proteins in cardiac muscle cells and thus important for contractility and cardiac function. Carnosine produces relaxation of isolated rat aorta independent of endothelium, this effect is partly mediated via cyclic GMP production and is not produced by its constituent amino acids, L-histidine and β-alanine.	(Roberts and Zaloga, 2000) (Ririe et al., 2000)
12.	Anti-ischemic activity	In brain and heart, anti-ischemic effect of carnosine is due to combination of antioxidant and membrane protecting activity, proton buffering capacity and formation of complexes with transition metals. In cerebral ischemia, carnosine decreases mortality and is beneficial in neurological conditions while in cardiac ischemia, carnosine protects cardiomyocytes from damage and improves contractility.	(Stvolinsky and Dobrota, 2000; Alabovsky et al., 1997)
13.	Enzyme regulation	Regulates muscle phosphorylases	(Johnson and Aldstadt, 1984)
14.	Calcium regulation	Carnosine acts as endogenous regulator of sarcoplasmic reticulum Ca release channel in skeletal muscles.	(Batrukova and Rubtsov, 1997)

2.7. Synthetic Carnosine versus Natural Carnosine

Carnosine can be chemically synthesized or extracted from skeletal muscles as a natural source by various extraction methods. Methods of chemical synthesis of carnosine and disadvantages of synthetic carnosine are discussed in this section.

Methods of Chemical Synthesis of Carnosine

A simple and efficient method of chemical synthesis of carnosine was suggested by Vinick and Stanley (1983). They used a coupling reaction between NTA N-(thiocarboxy) anhydride of β-alanine with L-histidine under controlled pH (8.1 to 9.4) and exploited the isoelectric point (8.2) of carnosine to obtain a 79% carnosine recovery. Carnosine can also be prepared synthetically using phthaloyl (Turner, 1953). In this method, phthaloyl-β-alanine chloride and L-histidine are condensed at lower temperatures in the presence of trimethylamine resulting in the formation of phthaloyl-β-alanylhistidine and later carnosine was isolated by detachment of phthaloyl group using hydrazine.

Disadvantages of Synthetic Carnosine

Synthetic preparation of pure carnosine contains hydrazines in the range of 0.01 to 0.20% (w/w) (Decker et al., 2000; Zhou et al., 1998). These levels are capable of interfering in analyzing antioxidant properties of carnosine since hydrazine also possesses antioxidant properties. Hydrazines are powerful reducing agents which can inactivate free radicals and accelerate metal promoted decomposition of lipid peroxides (Schmidt,

2001). Therefore, presence of hydrazines might lead to flawed interpretation when evaluating antioxidant properties of synthetic derivatives of carnosine.

Some Japanese pharmaceutical companies have preferred natural carnosine over synthetic carnosine for use in clinical trials due to the relatively low manufacturing cost and lower level chemical contaminants (hydrazines) (Quinn et al., 1992). Therefore, there is a desire for extraction of carnosine in the native form with minimal or no use of chemicals.

2.8. Extraction of Carnosine

The first systematic extraction of carnosine from beef extract was conducted by Guelwitsh in 1900 (Guelwitsch 1900; 1906; and 1911). Since then, carnosine has been extracted from a wide variety of animals; including buffalo, cat, chicken, beef, chum, crab, dolphin, dog, donkey, frog, Giant oyster, sheep, Siberian salmon, snake, sturgeon, squid, swine, trout, turkey, wallaby and blue whale. Table 2.2 provides a summary of the source and amounts of carnosine extracted from different animal muscles.

Carnosine and anserine extraction have been conducted from mammalian skeletal muscle (cat, dog, deer, gnu, oppossum and llama) by Wolff and Wilson (1935). Carnosine is present in as high as 20 mM in mammalian skeletal muscles and in lower levels in the central nervous system (Guiotto et al., 2005). Carnosine can be extracted from muscle tissues by simple hot water extraction or by use of acids such as hydrochloric acid, perchloric acid. According to the study conducted by Winnick et al (1963) using histidine ^{14}C and β-alanine ^{14}C radioactive isotopes, during extraction

more than 95% of radioactive peptides were present in the supernatant centrifuged at 15,000 g and negligible amounts were present in the pellet, indicating that centrifugation was an effective method for extraction of carnosine.

Carnegie et al (1983) used 0.9% saline and 8% sulfosalicyclic acid with centrifugation at 10,000 g and filtration to extract carnosine from meat; while heating of extract, centrifugation, and ultrafiltration technique were used on chicken extract (Maikhunthod and Intarapichet, 2005) and mechanically separated pork extract (Gopalakrishnan et al., 1999). Carnosine and total iron recovery increased with increased heating temperature in broiler thigh and breast meat (Maikhunthod and Intarapichet, 2005) while Gopalkrishnan et al. (1999) found that protein and iron content decreased with increases in temperature (60, 70, 80°C) in deboned pork extract. Maikhunthod and Intarapichet (2005) reported carnosine levels about 7 times higher in chicken breast (2900.1 μg/gm) than thigh (419.9 μg/gm) muscle. These researchers extracted carnosine at 60, 80, 100°C and with ultrafiltration (500 MW cut off). They also found that the 80°C ultrafiltrate had 20% higher carnosine but 40% lower protein levels and 10-30% lower iron concentrations than the 60°C heated ultrafiltrate; thus an increase in extraction temperature increased carnosine content while decreasing the mineral content.

Table 2.2: Source and amount of carnosine extracted/recovered from various muscle foods.

Muscle Type	Source	Amount	Reference
Beef	M. Semimembranosus	379mg/100gm of muscle	(Chan et al., 1993)
	Top side rump	333 mg/100gm tissue	(Carnegie et al., 1983)
	Shin	396mg/100gm tissue	
	Femoris	158mg/100gm	(Boldyrev et al., 1988)
	Myocardium	23mg/100gm	(Boldyrev, 1987)
Chicken	Breast	290mg/100gm	(Maikhunthod and Intarapichet, 2005)
	Breast	400mg/100gm	(Plowman et al., 1988)
	Breast	271mg/100gm of liquid extract	(Quinn et al., 1992)
	Thigh	42mg/100gm	(Maikhunthod and Intarapichet, 2005)
	Leg	124mg/100gm	(Plowman et al., 1988)
	Pectoralis	271mg/100gm	(Boldyrev, 1987)
Swine	Longissimus dorsi	240mg/100gm	(Easter and Baker, 1977)
	Shoulder and legs	276mg/100gm	(Carnegie et al., 1983)
	Loin and shoulder	466mg/100gm	(Gopalakrishnan et al., 1999)
	Deboned extract	105mg/100gm	
Crab	0	0	(Boldyrev and

			Severin, 1990)
Giant Oyster	0	0	(Boldyrev and Severin, 1990)
Sheep	Shoulder and leg Shoulder and leg Leg	111mg/100gm 173mg/100gm 190mg/100gm	(Carnegie et al., 1983)
Siberian Salmon	NI	0	(Boldyrev and Severin, 1990)
Turkey	Pectoral Leg Leg Breast	538mg/100gm 239mg/100gm 260 mg/100gm 240mg/100gm	(Wołos et al., 1982) (Davies et al., 1978)

The contents of the table were adapted from the review article by Chan and Decker (1994) and also additional carnosine values and references were added. Units are changed to mg/100gm (as the values indicated by the authors) for the uniformity of the table.

2.9. Antioxidant Activity of Carnosine

Oxidation of lipids occurs in three steps which are initiation, propagation and termination (Fenemma, 1996).

Step 1: Initiation can involve catalysts such as Fe^{2+}, Mn^{2+}, Co^{2+}, sunlight, another free radical or high energy sources to start the reaction that abstracts hydrogen from RH to form $R^{\cdot}$

$$RH + X^{\cdot} \longrightarrow R^{\cdot} + XH$$

$$RH + M^{III} \longrightarrow R^{\cdot} + H^{+} + M^{II}$$

(Metal ion)

Step 2: In propagation free radicals react to create peroxides and other free radicals

$R^{\cdot} + O_2 \longrightarrow ROO^{\cdot}$

$ROO^{\cdot} + RH \longrightarrow ROOH + R^{\cdot}$

Step 3: Termination, formation of stable end products.

$R^{\cdot} + R^{\cdot} \longrightarrow$

$R^{\cdot} + ROO^{\cdot} \longrightarrow$ Nonradical products

$ROO^{\cdot} + ROO^{\cdot} \longrightarrow$

Antioxidants are defined as the substances that can delay onset or slow the rate of oxidation (Fenemma, 1996). They can act by inhibiting the chain reaction.

$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot}$

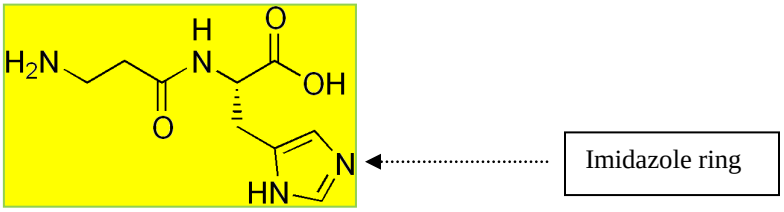
Antioxidants can be generally classified into four types based on the mechanism of action (Dziezak, 1986); (1) free radical scavenger, (2) peroxide decomposer, (3) metal chelator and (4) reactive oxygen scavenger. Other antioxidant-related effects include singlet oxygen quenching as with β-carotene, prevention of iron (Fe) cell membrane binding by zinc, activation of glutathione peroxidase activity by selenium and degradation of highly reactive hydrogen peroxide as with catalase.

Several researchers have reported various modes of antioxidant activity for carnosine. It acts as metal chelator, reactive oxygen scavenger, free radical scavenger and peroxide decomposer (Chan et al., 1993; Chan and Decker., 1994; Reddy et al., 2005; Baran, 2000). Carnosine protects against oxidation due to its reducing capacity (which is

related to breaking of radical chain by donation of a hydrogen atom), reactive hydroxyl radical scavenging and chelation of metal ions (Yen et al., 2002).

Various structure-function relationships of carnosine have also been published (Kohen et al., 1988; Chan and Decker., 1994; Baran, 2000) which indicates the imidazole ring of carnosine and the peptide linkage between histidine and β-alanine are responsible for its antioxidant capacity (Wu et al., 2003).

Figure 2.3: Carnosine Structure



Imidazole alone had 39% inhibition against peroxyl radicals and suggested that hydrogen on the methylene carbon next to imidazole ring is likely to be a proton donor which retards oxidation (Kohen et al., 1988). Chan and Decker (1994) supported the theory that the antioxidant properties of carnosine could also be attributed to the peptide bond present between β-alanine and histidine and not by histidine and β-alanine alone (Chan and Decker, 1994); similar results were found by Wu et al (2003).

2.10. Metal Chelating Activity of Carnosine

Carnosine acts as a metal chelator and prevents metal catalysts from initiating oxidation reactions. Carnosine forms a complex with copper, zinc, vanadium, nickel and

manganese ions (Baran, 2000). The protonated nitrogen (N^3) in the imidazole ring interacts with copper (Cu^{2+}) and zinc (Zn^{2+}) ions and thus makes a stable metal complex (Chan and Decker., 1994; Decker et al., 1992; Baran, 2000). Carnosine and anserine are excellent Cu^{2+} chelating agents. Carnosine has the ability to inhibit the oxidation of deoxyguanosine induced by ascorbic acid plus copper ions due to chelation of copper (Kohen et al., 1988).

2.11. Oxygen Radical Scavenging Capacity of Carnosine

The antioxidant activity of carnosine, homocarnosine and anserine against peroxyl radicals has been studied by Kohen et al. (1988) using voltametric measurements in AMVN {2,2-azobis (2,4-dimethylvaleronitrile)} and AAPH { 2,2'-azobis(2-amidino-propane dihydrochloride) }systems under physiological conditions. They also compared different structures such as carnosine, anserine, GABA {γ-amino butyric acid}, L-alanine, β-alanine and found that carnosine has 53% inhibition against peroxyl radicals while anserine showed 39% and GABA, L-alanine, β-alanine showed no inhibition. Histidine displayed 42% while histamine showed 28% inhibition against peroxyl radicals due to donation of hydrogen atom to the peroxyl radicals.

Carnosine is composed of β-alanine and L-histidine, and based on previous research studies histidine possesses stronger antioxidant properties. For instance, Chan and his colleagues in 1992 reported that histidine and histidine containing dipeptides were able to quench 49.1 to 94.9 % of hydroxyl radicals produced by Fe^{2+} and H_2O_2 (Chan et al., 1992). They used electron magnetic resonance spin trapping to determine that carnosine

was the most efficient inhibitor of hydroxyl radicals in phosphatidyl choline liposome emulsion followed by homocarnosine > histidine >gly-histidine >glycine-GABA > β-alanine (Chan et al., 1992).

During the study of carnosine inhibitory effect on the oxidation of lipids, proteins, deoxyribose oxidation and liposomes, Yen et al (2002) reported that carnosine stabilizes proteins against oxidation. Carnosine also inhibits the formation of protein carbonylation products which occurs due to oxidative stress on proteins (Aldini et al., 2005). Boldyrev et al (1990) and Yen et al (2002) studied the effect of carnosine, anserine, histidine and homocarnosine on luminol and lucigenin-dependent chemiluminescence of rabbit leukocytes which were activated by $BaSO_4$. These researchers found that anserine was most effective inhibitor followed by carnosine.

$$H_2O_2 + Cl_2^{\cdot} \longrightarrow OCl^{\cdot} + H_2O$$

Hydrogen Peroxide Chloride Hypochlorus anions water

Carnosine caused suppression of hypochlorus anions and formed stable chloramine complexes and in addition had an inhibitory effect on the myeloperoxidase enzyme itself.

2.12. Factors Affecting Antioxidant Capacity of Carnosine

Antioxidant capacity of carnosine could be affected by pH and temperature and the effect of these factors are discussed in detail in the following section.

2.12.1. Effect of pH on antioxidant activity

pH has a significant effect on the antioxidant activity of carnosine. A decrease in pH can cause a decrease in antioxidant activity however since carnosine acts as a buffer,

pH is maintained around the physiological range enabling carnosine to reduce oxidative stress in animal tissue (Kohen et al., 1988).

In 1990, Decker and Faraji found that the antioxidant activity of carnosine was unaffected over the pH range of 5.1-7.1 in meat and in aqueous solution while Boldrev and Severin (1990) found that the ability of carnosine to extend the lag phase of oxidation was decreased when pH was increased from 6.0. The relation between carnosine inhibition of lipid peroxidation and pH was also examined by Lee and Hendricks (1997). They found that at pH 6.0 the presence of carnosine reduced TBARS formation by two to three times greater than at pH 7.0. At the same pH, TBARS formation decreased as the concentration of carnosine increased from 0.02 mM to 20 mM.

2.12.2. Effect of heat on antioxidant activity

Heating carnosine at 100°C for 15 min had no effect on its ability to inhibit lipid oxidation (Decker and Faraji., 1990). Chan et al (1993) extracted carnosine from beef muscle using three treatments, unheated, 60°C and 100°C; and found that antioxidant activity of unheated muscle extract was not different from that of 60°C treatment while 100°C heating increased its antioxidant activity by 8.6 times. Increased antioxidant activity after heating at 100°C was due to removal of total iron (98% removed) from the extracts which act as pro-oxidants. They also found that antioxidant activity of the beef extract was affected little by freeze drying but was increased by vacuum drying. Carnosine extracted from chicken breast and thigh at 80°C had greater antioxidant activity than carnosine extracted at 60°C (Maikhunthod and Intarapichet,

2005). Similar results were found by Gopalakrishnan et al., (1999) in deboned pork extract exposed to (60, 70, 80°C) various temperatures. They suggested that with the increase in temperature, carnosine recovery was increased while iron recovery decreased which improved the net overall antioxidant activity of the extract due to less pro-oxidative compounds and greater carnosine extraction. Removal of pro-oxidants such as iron in order to increase the antioxidant activity was suggested by various scientists (Gopalakrishnan et al., 1999; Chan et al., 1993; Maikhunthod and Intarapichet, 2005).

2.13. Applications in Food

Carnosine may have applications as a natural antioxidant in foods, thus retarding lipid oxidation and retaining meat color during storage. Various scientists have explored the possible application of carnosine in food.

Decker et al (1991) found that carnosine is capable of preserving the red color of pork meat, thus can be used to improve retention of quality in fresh cut meat and also suggested that color stabilization was due to carnosine's antioxidant activity (Decker et al., 1995). Carnosine can also preserve meat color by preventing the formation of metmyoglobin (James et al., 1995).

Antioxidant activity of carnosine along with gamma irradiation of ground beef and beef patties was studied by Badr (2007). It was observed that carnosine significantly reduced oxidation and metmyoglobin formation in samples during storage. Badr (2007) concluded that carnosine can be used as a natural antioxidant to increase the oxidative stability of gamma irradiated raw and cooked meat products.

Djenane et al (2004) studied the antioxidant effect of carnosine (50mM), carnitine (50mM) and L-ascorbic acid solutions (500ppm) in fresh beef steaks stored under modified atmosphere. The combination of carnosine with ascorbic acid provided the best protection against oxidative deterioration when compared with carnosine, carnitine and ascorbic acid alone. The surface application of carnosine to meat delayed oxidation of meat more effectively than carnitine.

The effect of preblending of carnosine at levels 0, 0.5, 1 and 1.5% on the quality of ground buffalo meat under refrigeration (4 ± 1°C) was measured by Das et al (2006). Samples containing 1% and 1.5% carnosine significantly inhibited metmyoglobin formation and brown color development. Carnosine also increased water holding capacity, meat pH, cooking yield and oxidative stability when compared to control samples. These researchers concluded that 1% preblended carnosine with ground buffalo meat extended its shelf life by 8 days under refrigerated storage (Das et al, 2006).

Decker et al (1991) reported carnosine (0.5 and 1.5%) effectively inhibited formation of lipid peroxides and TBARS in frozen (− 15°C) salted ground pork for up to 6 months of storage. Carnosine (1.5%) was compared with sodium tri-polyphosphate (0.5%), α-tocopherol and butylated hydroxytoluene (0.02% of fat content) and found that inhibition of TBARS formation by carnosine was better than the other compounds tested. Moreover carnosine was the most effective in preventing oxidative rancidity and color changes as determined by sensory panel, in salted ground pork after 1 month of frozen storage (−15°C). In a similar study, Decker and Crum (1993) found antioxidant activity of carnosine in cooked salted and unsalted ground pork was greater than that of lipid-soluble

free radical scavengers, butylated hydroxytoluene and α-tocopherol but less than that of sodium tripolyphosphate. They concluded that carnosine could be used to reduce the oxidative deterioration of cooked salted and unsalted ground pork.

O Neill et al (1998) studied lipid oxidation in raw and cooked chicken thigh patties obtained from chicken fed with supplementary dietary α-tocopherols. They added carnosine (0-1.5%) into the patties and found that 1.5% carnosine provided the best antioxidant capacity in both raw and cooked patties up to 10 days and 7 days respectively, under refrigerated storage. These researchers also found that carnosine had a similar antioxidant effect as a dietary α-tocopherol supplement under refrigerated conditions and that α-tocopherols and carnosine acted synergistically having greater antioxidant activity together than when used singly. These same researchers (O'Neill et al., 1999) also found that carnosine (1.5%) inhibited both lipid oxidation as well as cholesterol oxidation in salted chicken thigh patties. Dietary supplementation of carnosine (0.5%) in live chicken increased breast and thigh muscle weight significantly and also significantly decreased TBARS values and increased total antioxidant capacity in meat.

Dietary supplementation of carnosine along with α-tocopherol was more effective in decreasing TBARS formation in rat skeletal muscle homogenate than carnosine alone, suggesting carnosine and α-tocopherol act synergistically preventing TBARS formation in skeletal muscle homogenate (Chan et al., 1994). Carnosine antioxidant activity and its effect on volatile compounds produced by lipid oxidation was studied in a meat model system by Kansci et al (1997). They induced oxidation by using Fe III and ascorbate 45

μM each. Carnosine (2-10 mM) showed a decrease in lipid oxidation, TBARS, c t-2-undecenal, total 2-alkenals and hexanol levels.

Meat ageing had no effect on carnosine and anserine concentrations in ready to eat beef meat while the concentration of these dipeptides decreased 82.6% and 76.0%, respectively, of their initial values after cooking at 75°C for 90min (Bauchart et al., 2006).

To summarize, carnosine was discovered from beef extract but a century after its discovery, little published research has been conducted relating to carnosine's possible microbicidal or food safety potential. Although it is known for flavor, color and antioxidant properties in foods, more work is needed to explore the use of carnosine as a shelf life extender of food products.

2.14. References

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CHAPTER THREE

EXTRACTION OF CARNOSINE FROM DIFFERENT POULTRY BY-PRODUCTS AND MEASURING ITS ANTIOXIDANT PROPERTIES

Abstract

The aim of the present research was (1) to extract carnosine from different poultry by-products and (2) to measure their antioxidant activities using different analytical methods. Poultry by-products such as head, liver, lungs, tail, gizzard, brain and heart were collected from a poultry processing facility. Carnosine was extracted using a hot water extraction and the content was analyzed using HPLC. Thiobarbituric Acid Reactive Species (TBARS) inhibition, metal chelating activity, free radical scavenging activity and Oxygen Radical Absorbing Capacity (ORAC) values were used as parameters to evaluate antioxidant activity of the extract. Carnosine was present in all the tissue samples investigated. Liver had the highest (102.29 mg/gm) and brain the lowest carnosine content (0.95 mg/gm) ($p \leq 0.05$). Except for the brain, all tissue ultrafiltrates and reconstituted dry powders showed TBARS inhibition ranging from 20.87-39.57% and 5.66 -14.47%, respectively. Head ultrafiltrate and reconstituted dry powder had the maximum while gizzard exhibited the minimum metal chelating activity ($p \leq 0.05$). Free radical scavenging activity of ultrafiltrate from all tissues samples ranged from 25.11 to 79.38%, while this activity was higher (29.76 to 84.05%) in the reconstituted dry powder of all tissue samples. ORAC values were highest in liver ultrafiltrate and lowest in heart ($p \leq 0.05$), with a similar trend in reconstituted samples with liver the highest and head the lowest ORAC values ($p \leq 0.05$).

Conclusions include that the carnosine was present in the poultry by-products ultrafiltrates, as well as its dry powder and it possesses antioxidant properties.

Abbreviations: ORAC = Oxygen Radical Absorbing Capacity; TBARS= Thiobarbituric Acid Reactive Species; TE= Trolox Equivalents

Keywords: Carnosine, antioxidants, TBARS, metal chelating, free radical scavenging, ORAC, poultry by-products

3.1. Introduction

More than a century ago in 1900, carnosine was extracted from Leibig's extract (beef extract) by Guelwitsh (Guelwitsch and Amiradgibi, 1900; Guelwitsch, 1906; Guelwitsch, 1911). Later, upon systematic analysis, it was determined that this water soluble dipeptide is composed of β-alanine and L-histidine and acts as a buffer in the muscle (Davey, 1960; Skulachev, 2000; Smith, 1938), a potent antioxidant in skeletal muscles (Chan and Decker., 1994; Kohen et al., 1988), a neurotransmitter in the brain (Trombley et al., 2000; Tomonaga et al., 2004; Tomonaga et al., 2005) and an aid in muscle contraction in skeletal tissues (Avena and Bowen, 1969; Severin et al.,1963). Carnosine also regulates calcium proteins in cardiac muscles (Roberts and Zaloga, 2000), exhibits anti-ageing effects (Hipkiss, 1998; Hipkiss and Brownson, 2000; Reddy et al., 2005) and chelates metal ions (Baran, 2000; Chan and Decker., 1994; Kohen et al., 1988). Carnosine has been proposed as a cure for Alziehmer's disease (Reddy et al., 2005), senile cataracts (Babizhayev et al., 2009), Wilsons disease (by chelation of Cu ions), gastric ulcers due to membrane protection activity (Matsukura and Tanaka, 2000), wounds and inflammation (Boldyrev and Severin, 1990; Nagai, 1980). In addition, oral intake of carnosine has been found to improve high intensity and exercise performance and endurance in humans (Sato et al., 2003). To summarize, since its discovery, extensive research has shown that carnosine is a potential therapeutic food and/ or dietary supplement.

Food scientists have interest in carnosine due to its antioxidant properties. Antioxidants are substances that retard rates of oxidation reactions when added to food

products. Antioxidants in food can be categorized as natural antioxidants (such as Vitamin E and ascorbic acid) and synthetic antioxidants [such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ)]. Synthetic antioxidants (BHA, BHT etc.) are strictly regulated by the FDA (Food and Drug Administration) due to lingering concerns about toxicity at elevated levels (Branen, 1975; Ito et al., 1986). Therefore, research has been conducted to identify natural antioxidants from different sources to minimize the need for synthetic antioxidants and to enhance the nutritional quality of food. Several researchers have reported that carnosine inhibits autooxidation in lipids and cell membranes by chelating metal ions, scavenging reactive oxygen, scavenging free radicals and by decomposing peroxides (Baran, 2000; Chan and Decker, 1994; Reddy et al., 2005; Decker and Faraji, 1990). Preventing oxidation of food products increases shelf life and maintains food quality for longer period of time. Oxidation of lipids in foods can lead to loss of sensory and nutritional quality and may also result in formation of toxic compounds posing health concerns such as atherosclerosis, cytotoxicity, mutagenesis and carcinogenesis (Gray et al., 1996). There is interest in carnosine as a natural antioxidant in the food industry, especially since there are no toxic effects associated with consumption of carnosine or its derivatives (Quinn et al., 1992; Sato et al., 2008) and excess carnosine is excreted through urine (Perry et al., 1967).

Since its isolation from beef extract, carnosine has been extracted from a variety of other animal tissues including mammalian skeletal muscle tissues of cat, dog, deer, gnu, oppossum, llama (Wolff and Wilson, 1935) and turkey (Davis et al., 1978).

Carnosine was found as high as 20 mM levels in mammalian skeletal muscles but in relatively lower levels in the central nervous system (Guiotto et al., 2005). Carnosine has also been extracted from mechanically deboned pork (Gopalakrishnan et al., 1999), poultry breast meat (Maikhunthod and Intarapichet, 2005) and from isolated muscle protein waste material (James et al., 1995).

According to a recent report in 2009, approximately 8.7 billion chickens were slaughtered in the United States resulting in production of 1.16 million metric tons of poultry by-product meal, 0.6 million metric tons of poultry fat and 0.5 million metric tons of feather meal (Swisher, 2009). At present, most of poultry by-products are being utilized by the rendering industry to produce protein meal and fat.

Little published information is available on extraction of antioxidants such as carnosine from poultry by-products. Therefore, the objective of the present research was (1) to isolate carnosine from different poultry by-products and (2) to determine its antioxidant properties using different analytical methods.

3.2. Materials and Methods

3. 2.1. Reagents

Trolox- [(±)- 6-hydroxy-2, 5, 7, 8- tetramethyl-chroman-2-carboxylic acid; CAS# 53118-07-1], ferrous chloride [CAS# 7758-94-3], ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"-disulfonic acid sodium salt; CAS#69898-45-9], TCA [trichloroacetic acid; CAS# 76-03-9], DPPH [2,2-diphenyl-1-picryl-hydrazyl; CAS# 1898-66-4],

fluorescein sodium salt [CAS# 518-47-8], 2-thiobarbituric acid [CAS# 504-17-6], L-carnosine [CAS # 305-84-0], BSA [bovine serum albumin; CAS# 9048-46-8], OPA [phthaldialdehyde reagent; Product # 057K5015], L-α-phosphatidylcholine Type IV-S [CAS# 8002-43-5], AAPH [2,2'- azobis (2-methylpropion-amidine) dihydrochloride 97% ; CAS # 2997-92-4] and TEP [3,3,3-tetarethoxypropane; CAS# 122-31-6] were purchased from Sigma Aldrich chemical company(St. Louis, Missouri, USA). Sodium acetate trihydrate [CAS# 6131-90-4], methanol- HPLC grade (0.2µm filtered) [CAS # 67-56-1], acetonitrile- HPLC grade (0.2µm filtered) [CAS # 75-05-8] were purchased from Fisher Scientific (Fair-lawn, New Jersey, USA). L-ascorbic acid sodium [CAS # 134-03-2] and 4-bromoaniline [CAS# 106-40-1] were purchased from Arcos Organics (New Jersey, USA) while BHT [butylated hydroxytoluene] CAS# 128-37-0 was purchased from MP Biomedical, Inc (Solon , Ohio, USA). All reagents were ACS grades or purer.

3.2.2. Sample Procurement and Preparation

Tissues samples from freshly slaughtered commercial broilers (approximately 6 weeks of age) were procured from a local poultry processing facility. Samples were packed in ice then transported to the laboratory. Upon arrival to the laboratory head and gizzard samples were prepared on the same day and packed in plastic bags and stored at -80°C± 2°C until further analysis. The other tissue samples were stored at -20°C± 2°C and prepared next day, packed in the plastic bags and stored at -80°C± 2°C until further analysis.

3.2.2.1. Preparation of Sample

Gizzard: Excess fat from the gizzard was removed. The gizzards were then cut open to remove any material followed by peeling off the inside thick lining. After peeling the thick lining (cutica gastrica), the gizzards were washed under lukewarm tap water to remove any additional adhering material, washed gizzards were dried using paper towel.

Head: The beak, comb, and skin were removed form each head using shears.

Brain: Brain samples were prepared from head by cutting open the skull using surgical scissors. Exposed brain tissues were removed using a spatula.

Tail: The skin, oil gland and adhering fat were removed to prepare the tails for grinding.

Liver: Liver was trimmed to remove excess fat layer and gall bladder if attached.

Heart: Fat and the epithelial membrane were removed from each heart.

Lungs: Any attached fat and bronchial tubes were removed from lungs.

All prepared tissues were ground using an America Eagle Meat Grinder (Model # AE-G12, Serial # 12SS912029, American Eagle Food Machinery Inc., Chicago, IL, USA). The ground tissue samples were packed in plastic bags and stored at -80±2°C until further analysis.

3.2.3. Extraction of Carnosine

Carnosine was extracted the method as described by Maikhunthod and Intarapichet (2005) with slight modifications (Figure 3.1). To one part of minced sample, two parts of pre-cooled (4°C) nano pure water was added and samples were homogenized in a blender (Ostersizer Model # 4937, Sunbeam Products Inc., Boca Raton, Florida, USA) using 4 cylcles of 2min each cycle with 2 min cooling with ice slush between each cycle (8 min total homogenization). The homogenate was centrifuged at 20,000 g for 30 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA). The supernatant was then filtered through Whatman #4 filter paper (GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA). The water extracted filtrate (supernatant) was subjected to a heat treatment at 80°C for 15 min in a temperature controlled water bath (Precision, Model#283, Thermo scientific, Winchester, VA, USA), followed by immediate cooling in an ice bath. The heated extract was centrifuged at 6000 g for 20 min to remove precipitated proteins. Supernatant was filtered through Whatman #4 filter paper.

The collected supernatant was then ultrafiltered using an Amicon Ultra-15 Filter device (Catalog# UFC900324, Millipore, Billerica, MA, USA) with a 3000 molecular weight cut off, using a centrifugal force of 4000g for 55 min at 14°C in a Beckman Model J-6B centrifuge (Beckman Coulter Inc., Brea, CA, USA). Ultrafiltration permeate was collected and divided into two portions; one portion of ultrafiltrate was stored at -80°C in plastic tubes until further analysis, while the second portion was freeze dried for

72 hr using a Virtis freeze dryer (Model # 6201-3220,Virtis Company Inc., Gardiner, NY, USA) and stored as a dried powder at -80 °C until further analysis.

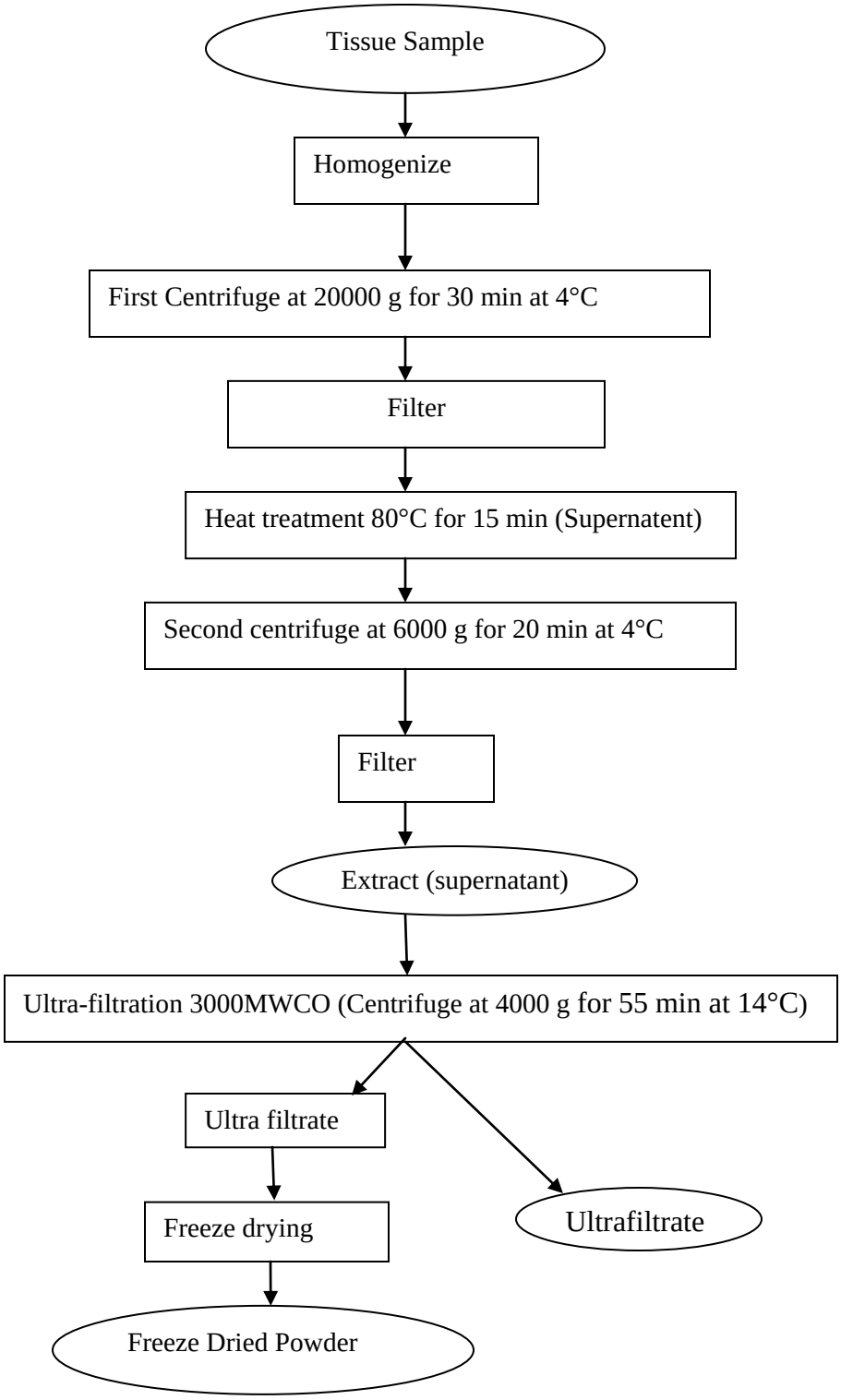

Figure 3.1: Flow Chart of Extraction Procedure

3.2.4. Determination of carnosine by Spectrophotometry

Spectrophotometric determination of carnosine was performed according to the method described by Parker, (1980). One ml of sample to be analyzed was placed in a glass tube and 1 ml of 0.04M EDTA, 1 ml of 20% Na_2CO_3 and 2 ml of diazotized p-bromoaniline were added. This mixture was vortexed for 10 seconds with Vortex Genie 2 ^{TM} (Model# G-560, Fisher Scientific, Bohemia, NY, USA). After 5 min, the reaction was stopped by adding 95% ethanol afterwhich absorbance was measured at 500nm using a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, NJ, USA). A standard curve was prepared using 20 to 100 μM pure carnosine plus above reagents. All reagents were prepared freshly before each use.

3.2.5. Determination of carnosine by HPLC

High pressure liquid chromatography (HPLC) measurement of carnosine concentration was conducted as described by Gopalakrishnan et al., (1999) and Maikhunthod et al. (2005) with slight modifications.

To 2 ml of liquid ultrafiltrate or freeze-dried extract (25 mg/ml, reconstituted in nano pure water), 2 ml of 0.4 M perchloric acid was added. The mixture was vortexed for 10 seconds (Vortex Genie 2 ^{TM,} Model# G-560, Fisher Scientific, Bohemia, NY, USA), boiled for 10 min (to precipitate proteins) and then centrifuged at 5000 g for 5 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA) . After centrifugation, the supernatant was filtered through 0.45

µm membrane filter. Filtrate was derivatized using phthaldialdehyde reagent (OPA) by adding 100 µl of OPA to 500 µl of sample, just prior to injection onto a HPLC (Shimadzu Instruments, Columbia, MD, USA).

OPA derivatized carnosine was separated using a mobile phase of 0.3 M sodium acetate (pH 5.5), methanol and acetonitrile (75:15:10) @ flow rate of 0.75 ml/min using Waters Spherisorb SCX-4.6 x 250 mm column (Waters Corporation, Milford, MA, USA). Derivatized carnosine was detected using a fluorescence detector (RF551 Spectroflurometric detector, Shimadzu Instruments) with excitation wavelength of 310 nm and emission wavelength of 375 nm. The standard curve was prepared using a standard carnosine solution (5 to 80 mM). The retention time and peak areas were analyzed by EZ start 7.4 Software provided with the equipment (Shimadzu Instruments, Columbia, MD, USA).

3.2.6. Antioxidant Activity Analysis

A multi antioxidant analytical approach was employed to better understand the antioxidant mechanisms and to evaluate overall antioxidant capacity (Di Bernardini et al., 2011; Zulueta et al., 2009; Frankel and Meyer, 2000). In the current study, the antioxidant activity of tissue sample (ultrafiltrate as well as reconstituted dry powder) was measured by thiobarbituric acid reactive species (TBARS) inhibition, oxygen radical absorbing capacity (ORAC), metal chelating activity and DPPH (2,2-diphenyl-1-picryl-hydrazyl) radical scavenging assay as described below.

3.2.6.1 Thiobarbituric acid reactive species (TBARS) Inhibition

TBARS measures malondiadehyde (MDA), the most abundant product of lipid oxidation and other secondary products of the reaction. TBARS inhibition was tested using a phosphatidyl choline emulsion system as described by Kansci et al (1997) and Gopalkrishnan et al. (1999). The TBA/TCA reagent was prepared by the method described by Tarladgis et al. (1960).

A 2 mg/ml phosphatidyl choline emulsion was prepared in 5 mM phosphate buffer at pH 7.0 using a Polytron® PT2100 homogenizer (Capitol Scientific Inc., Austin, Texas, USA). The phosphatidyl choline emulsion (1.8 ml) was mixed in 0.5 ml of sample and held at room temperature for 5 min for interaction of sample with the emulsion. After 5 min, emulsion oxidation was initiated using catalysts $FeCl_2$ (50 µl) and sodium ascorbate (100 µl) to the final concentration of $FeCl_2$ and sodium ascorbate of 40 µM each in emulsion system.

The polypropylene tubes (BD Falcon, Mississauga, ON, Canada) containing the mixture were incubated at 37°C in a temperature controlled water bath (Precison, Model#283, Thermo scientific, Winchester, VA, USA) and after 2 hours, the reaction was terminated by adding 50 µl of 10% BHT solution. To each tube, 2.5 ml of TBA/TCA solution was added and vortexed using a Vortex Genie 2 ^{TM} (Model# G-560, Fisher Scientific, Bohemia, New York) followed by heating in water bath at 90°C for 15 min. After heat treatment the tubes were cooled under running tap water and then

centrifuged at 5000 g for 15 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA).

Absorbance was read at 531 nm using a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, NJ, USA) and water was used as a blank. MDA (Malonaldehyde) was calculated using a standard curve prepared from TEP (1,1,3,3,-tetraethpxypropane) at concentrations from 0 to 70 nM MDA. All reagents were freshly prepared prior to experimentation. Emulsions without extract was used as a negative controls.

Percent TBARS Inhibition was calculated using following formula:-

{(MDA without extract-MDA with extract) / MDA without extract} x100

3.2.6.2. Free radical scavenging assay

Free radical scavenging assay was performed as described by Yen et al. (2002). DPPH radical (0.2mM) was dissolved in absolute ethanol. 2ml of ultrafiltrate or reconstituted dry powder samples were mixed with 2ml of DPPH. The mixture was allowed to stand for 30 min in dark and the absorbance of the resultant solution was measured at 517nm with a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, NJ, USA). Mixtures without extract were taken as negative controls and absolute ethanol was used as a blank.

Percent free radical scavenging was calculated as {(Absorbance of control at 517 nm – Absorbance of sample at 517 nm)/Absorbance of control at 517 nm} x 100

3.2.6.3. Metal Chelating Activity

Chelating activity on ferrous ions (Fe^{2+}) was measured using the method described by Yen and Wu (1999) with slight modifications. In this method, 1ml of ultrafiltrate or reconstituted dried powder (25mg/ml) was mixed with 3.6 ml of distilled water and the mixture was reacted with 200 µl of 2mM $FeCl_2$ and 200 µl of 5mM ferrozine for 20 min at ambient temperature. Absorbance was measured at 562nm using a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, NJ, USA). Nanopure water was used as a blank. Tubes containing no extract were used as a negative control for calculations and percent chelation was calculated using the following formula:-

{1- absorbance of the sample at 562 nm/ absorbance of control at 562 nm} x 100

3.2.6.4. Oxygen radical absorbing capacity assay

Oxygen radical absorbing capacity (ORAC) of the ultrafiltrate as well as of the reconstituted dried powder (25mg/ml) was determined by hydrophilic ORAC method described by Wu et al. (2008). The ultrafiltrate as well as reconstituted dried powder (25mg/ml) were diluted 100 times with phosphate buffer (pH 7.4).

Twenty five microliter of the diluted sample or Trolox standard solution (0, 10, 20, 40, 60,100µM) or blank (phosphate buffer pH 7.4) was added to one of 96 well plates (black with clear bottom, Optilux ™, BD Falcon). Flourescein solution (150µl) of 0.004 µM concentration was added to each well and the microplate was incubated at 37°C for

30 min in VWR-Incubating mini shaker (Model#980150, VWR, Arlington Heights, IL, USA). After 30 min incubation at 37°C, 25µl of AAPH solution (153mM) was added to each well (as the peroxyl generator) using auto injector (BioTek Instruments, Inc. Winnooski, Vermont, USA) to inititate the reaction. The microplate reader Synergy-HT from BioTek Instruments, Inc (Winnooski, Vermont, USA) was programmed to measure fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 520nm at 1min time intervals for 1 hr and ORAC values were calculated using software Gen5^{TM} (BioTek Instruments, Inc. Winnooski, Vermont, USA). The data were expressed as Trolox equivalents (TE) per gram of original sample (dry basis).

3.2.7. Protein Content

Tissue samples were analyzed for protein content by the Agricultural Services Laboratory at Clemson University using the Dumas method described in the Official Methods of Analysis, section 968.06 (AOAC, 1990) using nitrogen/protein analyzer (LECO Model# FP 528 ,Warrendale, PA, USA) .

3.2.8. Ash and Moisture Content

Moisture and ash content of the tissue samples was determined using official methods of analysis, section 900.02A and section 950.46B respectively (AOAC, 1990).

3.2.9. Total Mineral Content

Standard mineral contents in ultrafiltrate as well as reconstituted dried powder (25 mg/ml) were determined. Samples were diluted 1:10 for ultrafiltrate and 1:20 for reconstituted dried powder (25 mg/ml) before determining the mineral content. The samples were sent to Agricultural Services Laboratory at Clemson University where mineral content was determined using inductively coupled plasma spectrometer (SPECTRO ARCOS- ICP, Kleve, Germany).

3.2.10. Statistical Analysis

ANOVA was performed to determine overall differences in the group means. To determine specific differences between pairs of group means, the Fisher LSD test was used. Both tests used a Type I Error probability of 0.05 and the SAS (Statistical Analysis Software Edition 9.2, SAS Institute Inc., 2007) was used to perform the statistical calculations.

3.3. Results and Discussion

3.3.1. Time-temperature treatment for maximum recovery of carnosine

Maikhunthod and Intarapichet, (2005) reported a maximum recovery of carnosine from broiler breast and thigh meat using 80°C for 10 min. In our preliminary experimentation, the centrifuged filtrate (Figure 3.1) was subjected to three different

temperature-time treatments in order to obtain maximum recovery of carnosine from poultry by-products. These treatments were 80°C for 10 min., 80°C for 15 min., and 80°C for 20 min in a temperature controlled water bath. The carnosine content was determined using spectrophotometric methodology and there was no significant difference due to these time-temperature treatments on the recovery of carnosine ($p \geq 0.05$). Although not statistically different (Figure 3.2), individual observations for recovery of carnosine at 80°C for 15min were slightly higher than other treatments (data not shown). Therefore, 80°C for 15 min treatment was used to get maximum recovery in future experimentation.

3.3.2. Mass Balance and Proximate Composition

Weights from the initial step (original sample) to final step (dry powder) of carnosine extraction from by-products were recorded to calculate the mass balance of extraction procedure (Table 3.1). Based on an initial weight of 100 gm (constant), we found that liver provided the maximum yield of the powder while head provided lowest yield. Liver contains iron, bile salts and enzymes while head sample contain skull bone which plays a critical role during extraction or filtration step and thus affects the yield. Also the lipid content in the head versus liver might be another possible cause for lower recovery.

Among the group of tissues studied, dry solids were highest in tail tissue while lowest in lungs (Table 3.2). Protein content was highest in liver while lowest in brain. The ash content was highest in head and lowest in gizzard. The total mineral content of reconstituted dried powder was much higher than that of ultrafiltrate (Table 3.7 and 3.8).

3.3.3. Carnosine Content

Carnosine content determined by HPLC was highest ($p \leq 0.05$) in liver followed by lungs ($p \leq 0.05$); while the carnosine content of lungs was higher than heart, gizzard, tail, head and brain tissues ($p \leq 0.05$), and all these tissues did not differ ($p \geq 0.05$) in carnosine content (Table 3.2).

It was also observed that addition of OPA to prepared liver sample caused a change in color. This may have been due to a reaction between liver components and chemical reactants or due to the liver pH. A similar phenomena was observed in lung extracts but to lesser extent, the exact cause of this color change was not determined.

Maikhunthod and Intarapichet (2005) reported carnosine levels of 2900.1 μg/gm for chicken breast muscle and 419.9 μg/gm for thigh. These researchers extracted carnosine at 60, 80, 100°C for 10min, and using ultrafiltration (500 MWCO). They also found that 80°C ultrafiltrate had 20% higher carnosine but 40% lower protein levels and 10-30% lower iron concentrations than 60°C heated ultrafiltrate. All tissue samples in the present study had higher carnosine content in breast and thigh than those reported by Maikhunthod and Intarapichet (2005). This difference in carnosime recovery may be due to the different temperature-time treatments (85°C/15min) used during extraction procedure and due to discrepencies in detection methodology. Similar results were shown in our preliminary experimentation.

Carnosine was present in all the tissue samples studied, which agreed with previous researchers (Flancbaum et al., 1990) who measured carnosine in heart, kidney, stomach, muscle, liver, spleen, lung, ileum, hypothalamus, pituitary and olfactory bulb of

Hartley guinea pigs, mice and Spraguey-Dawley rats with the objective to determine carnosine role in histidine metabolism. They suggested that carnosine could be a reservoir for histidine-histamine metabolic pathway thus revealing another biochemical role of carnosine in addition to its antioxidant properties.

3.3.4. Antioxidant Activity Analysis

3.3.4.1. TBARS Inhibition

Thiobarbituric acid reactive species (TBARS) inhibition by liver ultrafiltrate was higher than that of other tissues ($p \leq 0.05$), while head, lungs gizzard, heart and tail did not differ ($p \geq 0.05$) (Table 3.3). In reconstituted dry powder (25 mg/ml), gizzard showed the highest antioxidant activity followed by liver, heart, head, lungs and tail (Table 3.4). The inhibition values of tail and gizzard reconstituted dry powder differed from that of other tissues ($p \leq 0.05$).

No TBARS inhibition was exhibited by brain ultra-filtrate as well as its reconstituted dry powder. TBARS inhibition of reconstituted dry powder of all tissue samples was lower than their respective ultrafiltrates (Figure 3.3). This lower activity may be due to higher mineral content in dry powder as compared to ultrafiltrate which can increase oxidation rate. Relative to carnosine content present, TBARS inhibition of head ultrafiltrate was 14 times greater than liver (Table 3.3). This indicates that there are other compounds present in the liver interfering with TBARS inhibition or acting as proxidants in the oxidation reaction. It is quite likely due to Fe present in liver which is

about 48 times more than head ultrafiltrate (Table 3.7); this Fe could acts as a catalyst in oxidation of phosphatidyl choline emulsion. It is also possible that there are other antioxidant compounds (Kohen, 1998) found in the brain including antioxidant enzymes as reported by Surai (1999).

Inhibition of TBARS by tissue ultrafiltrates as well as dry powder of the present study was less than the values of broiler's breast and thigh ultrafiltrate examined by Maikhunthod et al., (2005). This may be due to sample differences and also due to different concentrations of phosphatidyl choline emulsions used in the analyses as well as different concentration of catalysts ($FeCl_2$ and sodium ascorbate). TBARS inhibition of our tissue sample ultrafiltrates were higher while dry powder values were lower than the TBARS inhibition obtained from mechanically deboned pork extract (Gopalakrishnan et al., 1999).

Surai (1999) measured the activity of antioxidant enzymes superoxide dismutase, glutathione oxidase, catalase in kidney, lung, liver, brain, heart, and skeletal muscles (thigh) of chicken embryos and in one-day old chicks. All three enzymes exhibited activity in the different organs studied, but the level of activity varied among the different tissues. This study explained the ontogenic presence of antioxidant mechanisms during development and post-hatching. This study also explained the possible presence of antioxidant enzymes in the tissue sample of our study i.e. brain, liver, heart, lung and tail, and these enzymes might contribute to some antioxidant activity in our analytical methods.

Kohen (1998) also reported that the brain contains other antioxidants such as ascorbic acid (100 µM), urate (18 µM), homocarnosine (up to 50 µM), and anserine. O'Dowd et al., (1990) also confirmed the presence of N-acetyl forms of histidine, 1-methylhistidine, carnosine, anserine, and homocarnosine in rat brain using HPLC and NMR (Nuclear magnetic resonance) spectroscopy.

Tail tissue is composed of skeletal muscle, bone, cartilage and an oil gland. Skeletal muscles of chickens contain carnosine and anserine (Davis et al., 1978; Huang et al., 2000; Maikhunthod and Intarapichet, 2005). Although the amount of skeletal muscle in tail is much less than the breast and thigh this can contribute to carnosine content. Besides carnosine, tail (skeletal muscle) might also contain anserine, glutathione and polyamines such as spermine and spermidine which possess an activity similar to carnosine (Zhou and Decker, 1999).

Lung lining fluid contains extracellular and cellular glutathione peroxidase (Avissar et al., 1996) and in addition to these enzymes, skeletal muscles contain carnosine, anerine, and superoxide dismutase (Chan and Decker., 1994). Other hydrophilic antioxidants such as other small peptides like anserine, homocarnosine and uric acid, ascorbates might be present in the tissue samples (Sacchetti et al., 2008) . These water soluble molecules (amino acids and other small peptides) would be extracted with carnosine in our extraction process. Thus, it is quite likely that these substances along with carnosine (present in extract) contribute to the antioxidant activity of ultrafiltrate and reconstituted dry powder.

Furthermore, enzymes impart antioxidant activity similar to carnosine, such as superoxide dismutase, catalase or glutathione oxidase and glutathione reductase (Sacchetti et al., 2008; Chan and Decker, 1994). These enzymes can be partially denatured or lose activity by the temperature treatment used which could contribute to differences in activity. Carnosine is known to form complexes with certain divalent metal ions such as Co, Cu, Mn, Zn, Cd, while K, Mg, Ca, Na do not bind with carnosine (Boldyrev, 2007) The metal ions already present (Co, Cu, Mn, Zn, Cd) in the extract might promote oxidation reaction or act as proxidants (when added into the emulsion system of phosphatidyl choline) and thus, can lead to interference of the results.

As stated earlier, brain ultra-filtrate as well as reconstituted dry powder did not show TBARS inhibition. This may be due to ascorbic acid found in brain tissue reducing TBARS inhibition. According to Surai et al (1996), brain contained the greatest amount of ascorbic acid compared to liver, heart, lung and kidney. Since we used ascorbate and $FeCl_2$ as a catalyst for phosphatidyl choline emulsion oxidation, the ascorbates already present in the brain may act as proxidants synergistically with the catalysts used. The same authors (Surai et al., 1996) also found that in vitro incubation of brain homogenate with exogenous Fe^{2+} increased lipid peroxidation and thus, TBARS were much higher than liver homogenate. These results were in agreement with the present study.

3.3.4.2. Metal Chelating

Metal chelating activity of head ultrafiltrate was the highest followed by brain, liver, tail, lungs, heart and gizzard. Metal chelating activity of all tissues were different ($p \leq 0.05$) except brain and liver which were not different ($p \geq 0.05$) (Table 3.3).

In reconstituted dry powder (25 mg/ml), head showed the highest metal chelation followed by lungs, tail, heart, liver, brain and gizzard (Table 3.4). Metal chelating activity of heart in comparison to lung and liver was not different ($p \geq 0.05$) while other tissues were different ($p \leq 0.05$). Metal chelating activity of reconstituted dry powder of liver and head was lower than its ultrafiltrate while in the rest of the tissue samples, the dried powder showed more metal chelation than their ultrafiltrates (Figure 3.4).

Carnosine acts as a metal chelator and stops the oxidation reactions (Baran, 2000) and it forms a ligand complex with Fe^{2+} in similar manner as with Cu^{2+} (Huang et al., 2005). Relative to the carnosine content presence, head ultrafiltrate showed 22 times more metal chelation than liver. This could be due to 20 times more iron already present in the liver (3.6ppm) utrafiltrate than head (0.18ppm) (Table3.7). The presence of iron could also promote color development during the assay by forming a complex with ferrozine interfering with the assay results. Besides carnosine, there are other dipeptides such as anserine which also plays a role in metal chelation and amino acids (such as histidine) which form complexes with Fe^{2+} resulting in mixed ligand formation (Boldyrev, 2007) changing metal chelation values. Huang (2000) also found that demineralized breast extract gave higher metal (ferrous ion) chelation than un-mineralized extract. Therefore, as suggested by other scientists, removal of proxidants

such as iron helps increase the antioxidant activity of the ultrafiltrate (Chan et al., 1993; Gopalakrishnan et al., 1999; Maikhunthod and Intarapichet, 2005). So for better evaluation of the metal chelation and TBARS inhibition, the demineralization of the extract is a possible option however, this would add cost to process if commercialized.

3.3.4.3. Free Radical Scavenging

Ultrafiltrate of liver had the highest scavenging of DPPH radical followed by tail, lungs, gizzard, brain, head and heart (Table 3.5). Except for tail and liver, the free radical scavenging activity of all other tissue ultrafiltrates were significantly different ($p \leq 0.05$).

Free radical scavenging of reconstituted dry powder was the highest in liver followed by tail, heart, gizzard, lungs, head and brain (Figure 3.5). The values of tail and heart as well as lungs and gizzard did not differ ($p \geq 0.05$); all other values were significantly different. Except brain, all tissue samples reconstituted dry powder exhibited more free radical scavenging than their respective ultra-filtrates (Figure 3.5).

The inhibition of DPPH radical observed by Huang et al., (2000) in breast and thigh extract was 71.0%. Some of our tissue samples (both ultrafiltrate and reconstituted dry powder) showed higher values than those observed by these authors while some tissue samples showed lesser values. When compared to the individual tissues samples with respect to carnosine content presence, tail showed 18 times more free radical scavenging activity than liver which implied that liver may contain other compounds which interefere with the free radical scavenging ability of carnosine. In tail, other than carnosine, aromatic amino acids (such as histidine, tyrosine, tryptophan) can donate

protons or electrons to deficient radicals terminating the oxidation reaction. Cysteine, non-aromatic amino acid, has direct interaction with radicals (Sarmadi and Ismail, 2010). Polyamines also cause radical scavenging activity (Sacchetti et al., 2008) .

3.3.4.4. ORAC Values

ORAC assay is based on hydrogen atom donation by antioxidants to stabilize peroxyl radicals produced by AAPH. In this method, the quantification is obtained from kinetic curves derived from competitive kinetic reactions (Huang et al., 2005). ORAC was highest in liver ultarfiltrate followed by gizzard, lungs, brain, head, tail and heart (Table 3.5). ORAC values of heart, head and tail as well as lungs and gizzard did not differ ($p \geq 0.05$) while rest of the tissues were different ($p \leq 0.05$).

In reconstituted dry powder, ORAC values were the highest in liver followed by lungs and gizzard, heart, brain, tail and head. Except brain and heart, ORAC values of all other tissues samples differed ($p \leq 0.05$) (Table 3.6). Relative to carnosine presence, ORAC values of gizzard were about 5 times more than liver, which implied that even though liver is high in carnosine, the hydrogen donating ability of liver carnosine might be interefered by other components. Overall, ORAC values of reconstituted dry powder were less than their respective ultrafiltrates (Figure 3.6) which indicates that there is overall loss in ORAC due to drying.

ORAC values of tissue samples studied were higher than hydrophilic extract of beef samples obtained by Wu et al (2008). Kohen et al (1988) determined antioxidant activity of carnosine, homocarnosine and anserine against peroxyl radicals by using

voltametric measurements in AMVN {2,2-azobis (2,4-dimethylvaleronitrile)} and AAPH { 2,2'-azobis(2-amidino-propane dihydrochloride) }systems under physiological conditions. They found that carnosine has 53% inhibition against peroxyl radicals while anserine showed 39% and GABA {γ-amino butyric acid}, L-alanine, β-alanine showed no inhibition. Histidine displayed 42% while histamine showed 28% inhibition against peroxyl radicals due to donation of hydrogen atom to the peroxyl radicals. Our samples might contain other dipeptides such as anserine, homocarnosine which interact with peroxyl radicals in the same way as carnosine thus affecting the ORAC antioxidant capacity.

Overall, carnosine is present in the tissue investigated and all the tissues sample ultrafiltrates as well as reconstituted dry powder showed TBARS inhibition except brain. All the samples showed metal chelating, free radical scavenging and ORAC values. With the freeze drying process, all the sample lose their antioxidant activity about 2 fold or even more, so alternate method of drying such as vacuum drying could be explored.

3.4 **Conclusions**

The main objective of this study was to extract carnosine from the poultry by-products, which could increase the revenue of the poultry processing or the rendering industry. Carnosine was present in the poultry by-products ranging from 0.95 to 102.29 mg/gm sample (wb) and showed excellent antioxidant properties. Carnosine extraction from poultry byproducts using hot water could be a cost-effective method and could

produce excellent profit margins for manufacturers. There is an increasing demand of producing novel functional foods containing bioactive peptides such as carnosine, anserine and L-carnitine etc. (Arihara, 2006). Hence, extracted carnosine could be used in formulated foods or in nutritional supplements for animals including pets with increased therapeutic and nutritional values.

3.5. References

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TABLES AND FIGURES

Table 3.1: Mass balance of extraction process of carnosine from different organ samples (extract, ultrafiltrate and freeze dried ultrafiltrate)

Organ	Sample wt. (gm)	Supernatant (gm)	Pellet (gm)	Ultra filtrate (gm) [3]	Oversize matter (gm)	Freeze dried powder (gm) Yield [4]
Tail	100	135.57±5.18	146.67±3.52	116.60±2.56 (406.93)	18.97±2.56	0.96±0.21 (0.12)
Gizzard	100	174.29±6.84	120.50±14.46	166.33±0.97 (475.05)	7.96±0.98	1.26±0.16 (0.18)
Liver	100	190.67±2.37	90.56±3.60	184.27±3.15 (11946.22)	3.67±0.21	3.88±0.32 (10.41)
Head	100	155.87±14.31	144.38±3.81	106.95±2.18 (337.37)	51.68±0.22	0.57±0.08 (0.11)
Brain	100	170.35±1.75	111.73±1.78	79.59±7.15 (349.81)	101.25±1.24	0.61±0.07 (0.09)
Lungs	100	205.67±9.85	78.94±6.22	170.39±1.99 (1880.18)	29.18±6.26	1.18±0.38 (1.46)
Heart	100	197.31±4.84	81.21±3.04	180.49±2.67 (920.84)	16.81±2.66	1.34±0.02 (0.21)

1. Mean± SEM (N =3)
2. Two parts of nano pure water was added to each part of original sample weight.
3. Values in the ultrafiltrate column parenthesis indicate mg of carnosine present per 100 gm of original tissue sample.
4. Values in the freeze dried column parenthesis indicate mg of carnosine present per 100 gm of original sample.

Table 3.2: Proximate composition: Organ Samples

	Dry Solids (%)	Moisture Content (%)	Protein content (%)	Ash content (%)	Carnosine content (wb) [1]	Carnosine content (db)
Tail	42.60 ± 0.71	57.40± 0.71	17.10±1.89	1.95±0.342	1.16±0.28 [c]	2.61±0.65 [c]
Gizzard	19.99 ± 0.13	80.01 ± 0.13	17.6±1.06	0.76±.007	1.76±0.009 [c]	8.82±0.05 [c]
Liver	29.70 ± 0.11	70.30 ±0.11	19.8±0.64	1.29±0.014	102.29±1.96 [a]	397.26±7.58 [a]
Head	29.04 ± 0.44	70.96 ± 0.44	14.10±0.31	5.56±0.254	1.09±0.05 [c]	3.75±0.16 [c]
Brain	18.64 ± 0.25	81.36 ± 0.25	10.7±0.96	1.58±0.135	0.95±0.35 [c]	2.92±0.3 [c]
Lungs	17.78 ± 0.33	82.22 ± 0.33	13.2±0.46	0.88±0.030	14.41±0.73 [b]	81.05±4.10 [b]
Heart	26.04 ± 0.58	73.96 ± 0.58	16.0±1.22	0.96±0.071	2.07±0.16 [c]	7.96±0.58 [c]

1. HPLC method was used for determination of carnosine content; carnosine content is expressed in mg/gm of original sample on dry weight basis. Wb= wet basis and db= dry basis.
2: All values are Mean ± SEM (N=3)
3: Fisher's Least Significant Difference Test was used to compare mean values; [a-c] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).

Table 3.3: TBARS inhibition and metal chelating activity of ultrafiltrate

Sample	Carnosine content (mg/ml)	TBARS Inhibition [1] (%)	Metal Chelating Activity [2] (%)
Tail	3.44±0.75 [3]	20.87±2.55 (6.06) [4, b]	71.97±0.49(20.92) [c]
Gizzard	4.02±0.67	26.52±0.85(6.59) [b]	23.54±0.83(5.85) [f]
Liver	64.11±0.16	39.57±1.53(0.61) [a]	85.99±0.91(1.34) [b]
Head	3.11±0.01	27.59±1.85(8.87) [b]	94.81±0.39(30.48) [a]
Brain	4.32±0.46	No activity detected	86.59±0.94(20.04) [b]
Lungs	10.89±0.10	26.67±0.97(2.44) [b]	53.35±0.92(4.89) [d]
Heart	5.03±0.36	20.91±2.25(4.15) [b]	45.36±1.52(9.02) [e]
Carnosine	2.39±0.06	11.09±2.76(4.64) [c]	94.93±4.6 (39.72) [a]

1: % TBARS inhibiton = {(MDA without extract-MDA with extract) / MDA without extract} x100
2: % Metal chelating activity= {1- absorbance of the sample at 562nm/ absorbance of control at 562nm} x 100
3: All values are Mean ± SEM (N=4)
4: Values in the brackets are comparisons of antioxidant test value to carnosine content of the respective tissue sample.
5: Fisher's Least Significant Difference Test was used to compare mean values; [a-c] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).
6: Carnosine was used as standard for comparison.

Table 3.4: TBARS inhibition and metal chelating activity of reconstituted dry powder (25mg/ml)

Sample	Carnosine content (mg/ml)	TBARS Inhibition [1] (%)	Metal Chelating Activity [2] (%)
Tail	3.02±0.74 [3]	5.66±0.80(1.87) [4, c]	82.63±2.68(27.36) [b]
Gizzard	3.49±0.02	14.47±1.94(4.14) [a]	60.74±2.07(17.40) [e]
Liver	66.29±1.26	11.31±2.01(0.17) [a, b]	76.60±0.59(1.15) [c]
Head	4.78±0.20	10.49±1.23(2.19) [a, b, c]	92.20±0.42(19.28) [a]
Brain	3.91±1.45	No activity detected	68.67±5.14(17.56) [d]
Lungs	30.53±1.54	8.21±1.54(0.26) [b, c]	83.91±1.36(2.75) [b]
Heart	3.86±0.28	10.72±1.51(2.77) [a, b]	80.32±0.83(20.80) [b, c]
Carnosine	2.39±0.06	11.09±2.76(4.64)[a, b]	94.93±4.67 (39.72) [a]

1: % TBARS inhibition = {(MDA without extract-MDA with extract) / MDA without extract} x100
2: % Metal chelating activity= (1- Absorbance of sample at 562nm)/ Absorbance of control at 562nm * 100
3: All values are Mean ± SEM (N=4)
4: Values in the brackets are comparisons of antioxidant test value to carnosine content of the respective tissue sample
5: Fisher's Least Significant Difference Test was used to compare mean values; [a-c] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).
6: Carnosine was used as standard for comparison.

Table 3.5: Free radical scavenging and ORAC values of ultrafiltrate

Sample	Carnosine content (mg/ml)	Free Radical scavenging [1] (%)	ORAC values [2] (Trolox Equivalents)
Tail	3.44±0.75 [3]	77.71±1.15(22.59) [4, a]	38.34±1.45 (11.15)[d]
Gizzard	4.02±0.67	61.40±0.94(15.27) [c]	101.21±10.46 (25.18)[b]
Liver	64.11±0.16	79.38±0.45(1.24) [a]	303.11±0.82 (4.73) [a]
Head	3.11±0.01	30.59±0.19(9.83) [e]	39.50±4.34 (12.70) [d]
Brain	4.32±0.46	57.77±0.84(13.37) [d]	62.87±4.81(14.55) [c]
Lungs	10.89±0.10	74.57±0.27(6.85) [b]	100.57±12.17 (9.24)[b]
Heart	5.03±0.36	25.11±1.07(4.99) [f]	33.54±5.05 2(6.67)[d]
Carnosine	2.39±0.06	8.92±1.75(3.73) [g]	-

1: % Free radical scavenging was calculated as {(Absorbance of control at 517 – Absorbance of sample at 517) /Absorbance of control at 517} x 100
2: ORAC Assay (Oxygen Radical Absorbing Capacity); Values are expressed in Trolox equivalents (TE) per gram of original sample (dry basis).
3: All values are Mean ± SEM (N=4)
4: Values in the brackets are comparisons of antioxidant test value to carnosine content of the respective tissue sample
5: Fisher's Least Significant Difference Test was used to compare mean values; [a-g] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).
6: Carnosine was used as standard for comparison.

Table 3.6: Free radical scavenging and ORAC values of reconstituted dry powder (25mg/ml)

Organ	Carnosine content (mg/ml)	Free Radical scavenging [1] (%)	ORAC values [2] (Trolox Equivalents)
Tail	3.02±0.74 [3]	78.43±1.43(25.97) [4, b]	21.77±0.33(7.21) [e]
Gizzard	3.49±0.02	74.04±0.23(21.21) [c]	71.49±3.91(20.48) [c]
Liver	66.29±1.26	84.05±0.45(1.26) [a]	232.08±6.02 (3.50) [a]
Head	4.78±0.20	67.59±0.16(14.14) [d]	15.76±0.19 (3.30) [e]
Brain	3.91±1.45	29.76±0.68(7.61) [e]	33.23±0.89 (8.49)[d]
Lungs	30.53±1.54	73.24±1.06(2.39) [c]	86.88±4.70 (2.85)[b]
Heart	3.86±0.28	77.78±0.39(20.15) [b]	35.72±0.95(9.25) [d]
Carnosine	2.39±0.06	8.92±1.75(3.73) [f]	-

1: % Free radical scavenging was calculated as {(Absorbance of control at 517 – Absorbance of sample at 517)/Absorbance of control at 517} x 100

2: ORAC Assay (Oxygen Radical Absorbing Capacity); Values are expressed in Trolox equivalents (TE) per gram of original sample (dry basis).

3: All values are Mean ± SEM (N=4)

4: Values in the brackets are comparisons of antioxidant test value to carnosine content of the respective tissue sample

5: Fisher's Least Significant Difference Test was used to compare mean values; [a-f] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).

6: Carnosine was used as standard for comparison.

Table 3.7: Mineral Composition of ultrafiltrate

Sample	Mineral Composition (ultrafiltrate) pm											
	P	**K**	**Ca**	**Mg**	**Zn**	**Cu**	**Mn**	**Fe**	**S**	**Na**	**B**	**Al**
Tail	383.46	1021.65	45.34	37.28	0.08	0.07	0	0.03	113.89	364.02	0.09	0.01
Gizzard	237.98	903.55	18.11	27.83	0.52	0.01	0.08	0.14	146.46	300.51	0.07	0
Liver	529.17	1022.51	10.59	48.45	0.95	0.03	0.44	2.89	223.93	332.30	0.05	0
Head	134.64	604.74	25.14	16.11	0.04	0.17	0	0.06	167.9	461.14	0.07	0
Brain	265.01	850.09	10.39	18.39	0.03	0.07	0	0.07	68.24	405.3	0.1	0.02
Lungs	172.89	501.68	9.48	16.02	0.09	0.06	0.03	0.11	115.61	279.26	0.05	0
Heart	251.19	757.58	8.75	30.07	0.07	0.03	0.02	0.16	270.17	403.15	0.06	0

Table 3.8: Mineral Composition of reconstituted dry powder

Sample	Mineral Composition (reconstituted dry powder) ppm											
	P	**K**	**Ca**	**Mg**	**Zn**	**Cu**	**Mn**	**Fe**	**S**	**Na**	**B**	**Al**
Tail	760.32	1831.1	84.66	71.4	0.12	0.08	0	0.08	204.32	758.12	0.18	0
Gizzard	603.94	2153.52	36.46	63.82	1.28	0.2	0.2	0.82	351.66	748.88	0.22	0.04
Liver	482.18	877.78	8.78	40.88	0.64	0.02	0.36	3.6	196.18	322.02	0.1	0
Head	556.02	1813.44	53.6	57.06	0.14	0.32	0.04	0.18	562.04	1386.92	0.26	0.02
Brain	683.80	1900.70	26.7	45.2	0.12	0.16	0.02	0.1	156	1009.74	0.18	0
Lungs	733.44	1944.34	39.84	64.74	0.38	0.3	0.22	0.88	405.66	996.38	0.22	0
Heart	700.20	2014.62	22.98	77.98	0.28	0.12	0.08	0.48	682.04	1111.4	0.18	0

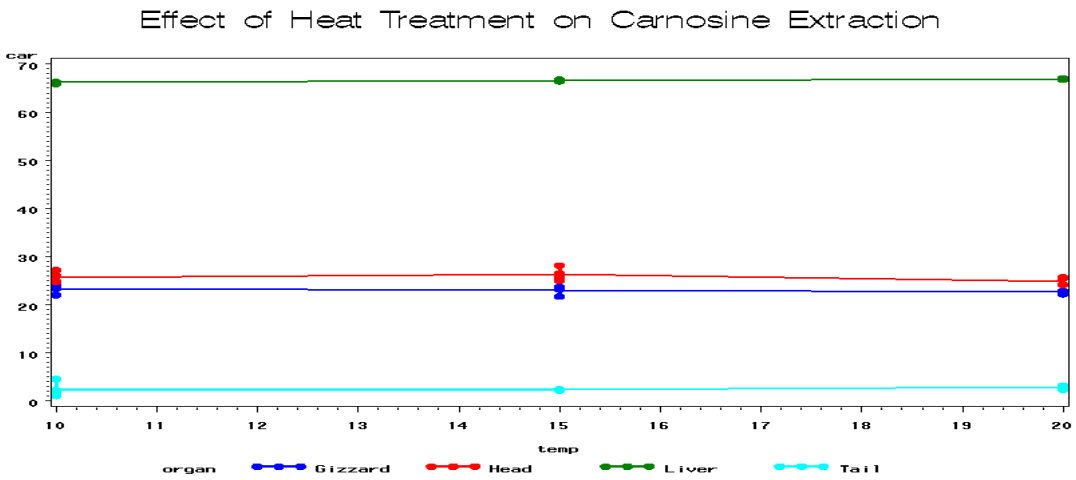

Figure 3.2: Time-temperature treatment for the maximum carnosine recovery. Gizzard, head, liver and tail was used to extract carnosine (N=5). Time temperature treatment 80°C/10min, 80°C/15min, and 80°C/20 min was given. Treatment mean values are not significantly different ($p \geq 0.05$).

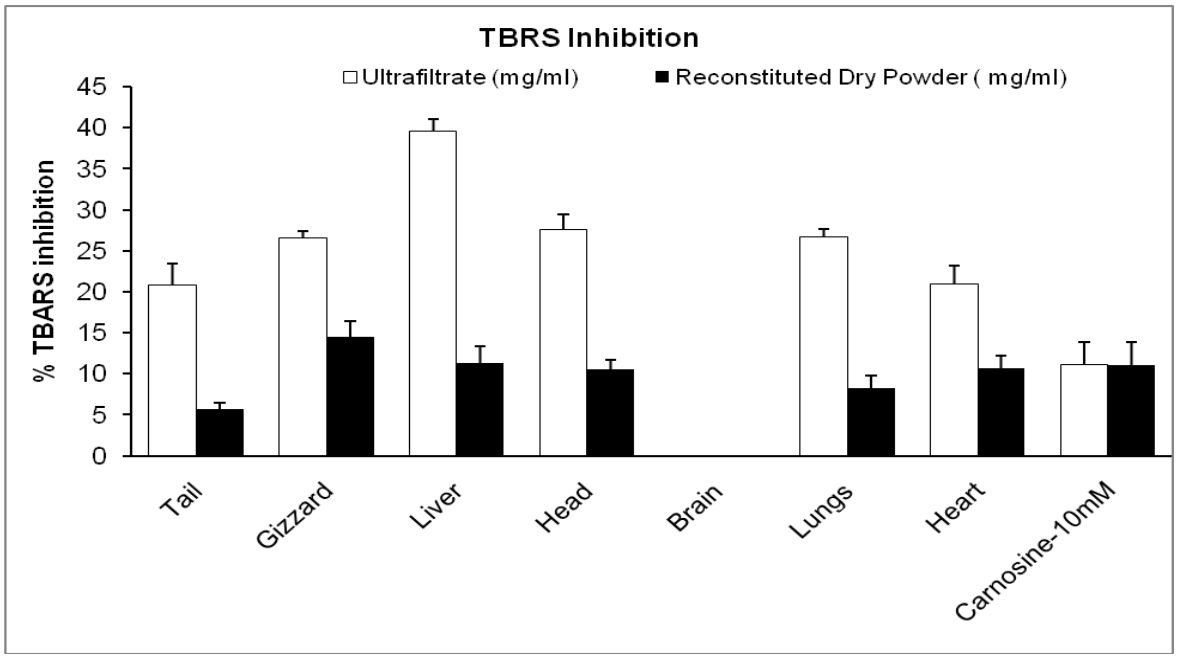

Figure 3.3: Comparison of TBARS inhibition of ultrafiltrate as well dry powder. Data given as Mean± SEM (N=4). All tissue samples ultrafiltrate and reconstituted dry powder TBARS values were significantly different ($p \leq 0.05$). Carnosine was used as standard for comparison.

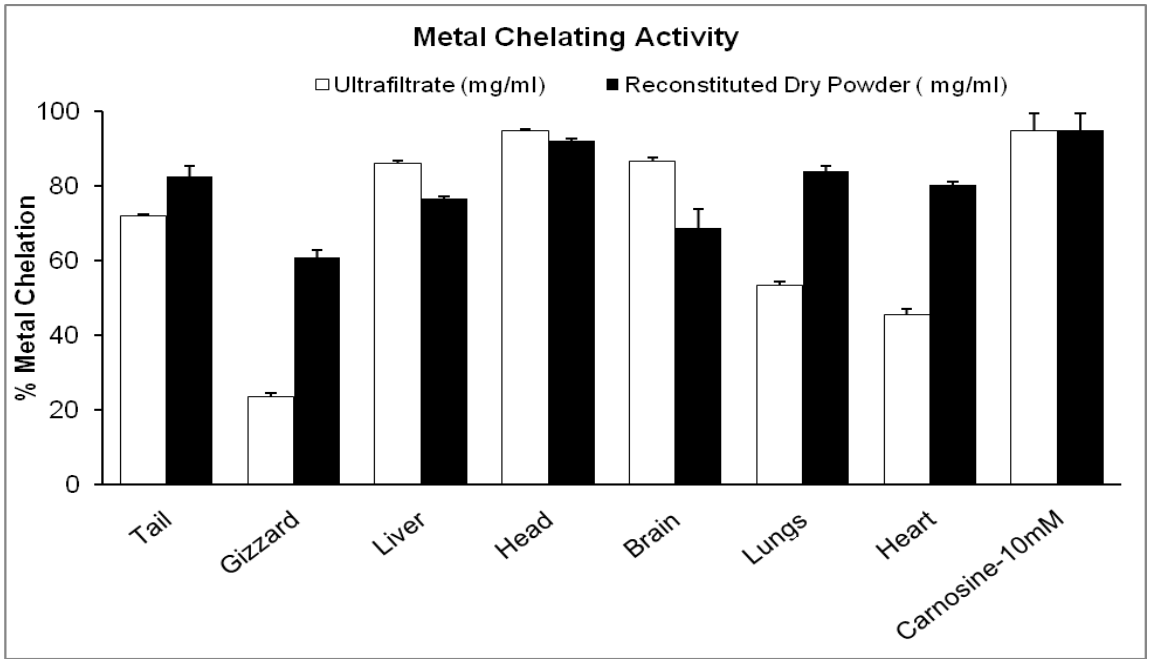

Figure 3.4: Comparison of Metal chelating activity of ultrafiltrate as well as dry powder. Data given as Mean± SEM (N=4). Metal chelating activity of ultrafiltrate and reconsituted dry powder were significantly different ($p \leq 0.05$). Carnosine was used as standard for comparison.

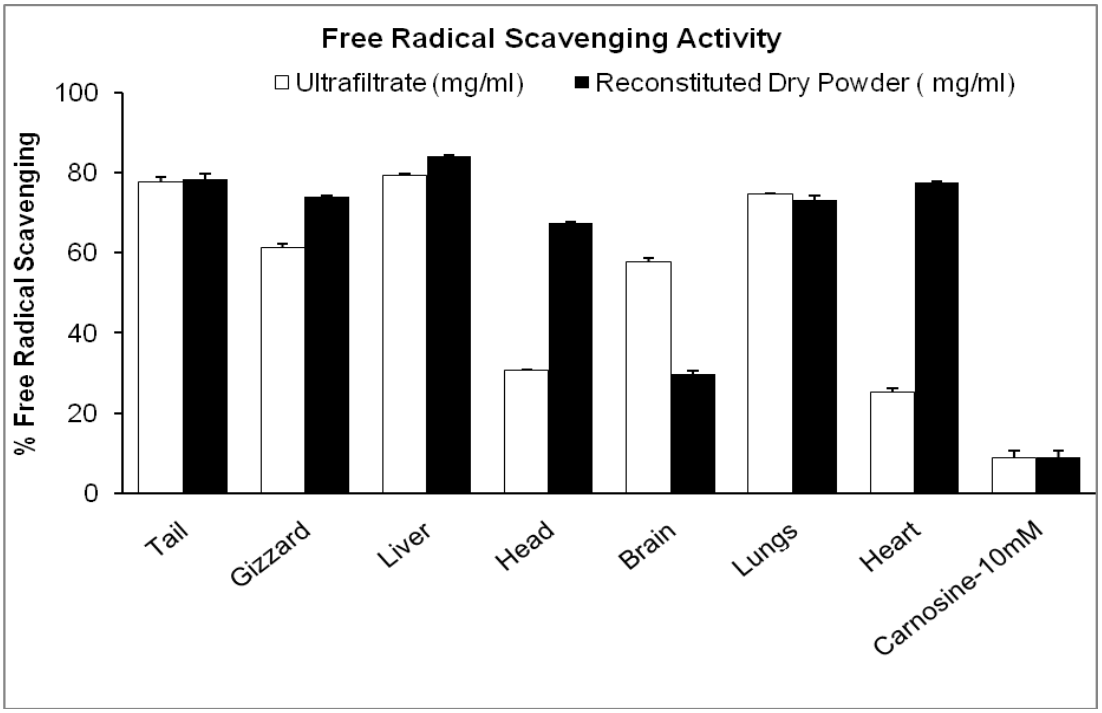

Figure 3.5: Comparison of free radical scavenging activity of ultrafiltrate as well dry powder. Data given as Mean± SEM (N=4). Free radical scavenging activity of ultrafiltrate and dry powder were significantly different ($p \leq 0.05$). Carnosine was used as standard for comparison.

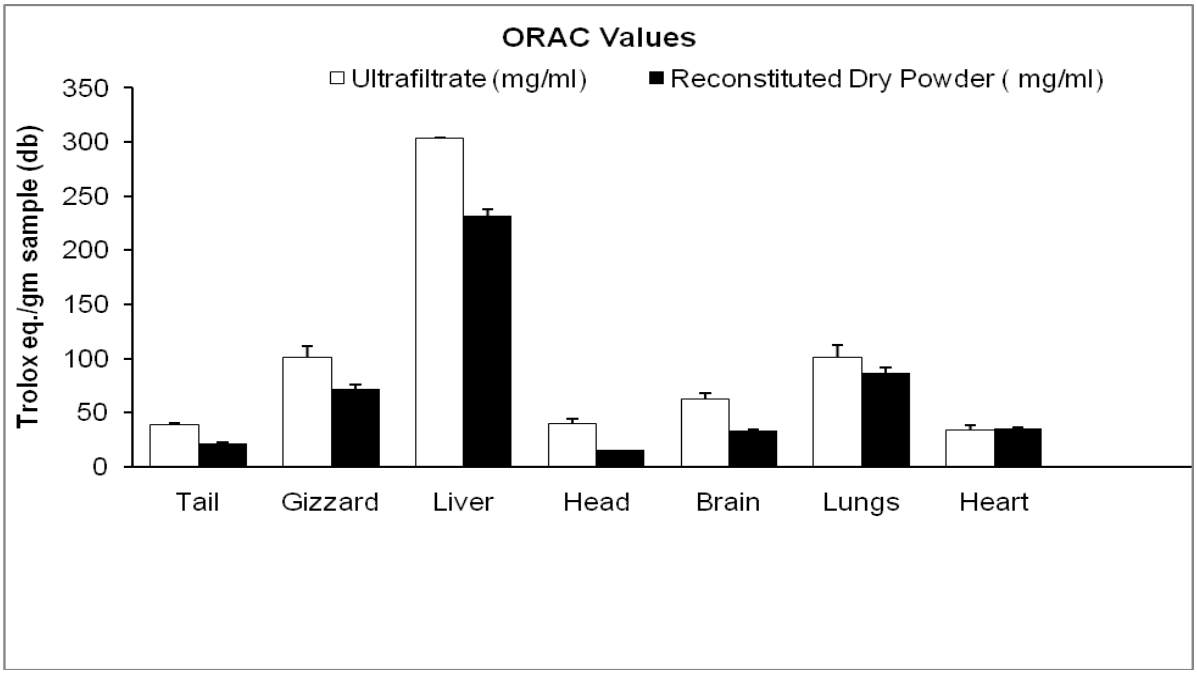

Figure 3.6: Comparison of ORAC values of ultrafiltrate as well as dry powder. Data given as Mean± SEM (N=4). ORAC values of heart ultrafiltrate and reconstituted dry powder were not significantly different ($p \geq 0.05$); while all other tissue samples ultrafiltrate and reconstituted dry powder ORAC values were significantly different ($p \leq 0.05$).

CHAPTER FOUR

CARNOSINE PRESENCE IN POULTRY PROTEIN MEALS AND ITS ANTIOXIDANT CAPACITY

Abstract

The aim of this research was: (1) to determine if carnosine is present in poultry protein meals and (2) to determine its antioxidant properties using different methods. Sample-A and sample-G were two poultry protein meal samples obtained from two different rendering facilities. Carnosine was extracted using hot water and analyzed by HPLC. Antioxidant properties of extracted carnosine were evaluated using TBARS inhibition, metal chelating activity, free radical scavenging activity, and ORAC assay. Carnosine content of sample-G was almost 2.6 times higher (104.71 mg/100g of dry sample) than sample–A (40.28 mg/100g of dry sample) ($p \leq 0.05$). TBARS inhibition by sample-G was 15.86% while sample-A did not exhibit any TBARS inhibition. Metal chelating activity and free radical scavenging activities of sample-A and sample-G did not differ ($p \geq 0.05$); sample-A (64.16% and 81.41%) and sample-G (63.78% and 84.17%) respectively. ORAC values (µM TE /gm of dry sample) of sample-A (84.35) were greater than sample-G (68.44) ($p \leq 0.05$). Overall, carnosine was present in poultry protein meals, and retained its antioxidant properties after high temperature rendering (~115°C).

Abbreviations: ORAC = Oxygen Radical Absorbing Capacity; TBARS= Thiobarbituric Acid Reactive Species; TE= Trolox Equivalents

Keywords: Carnosine, Poultry protein meal, antioxidant, ORAC, TBARS

4.1 Introduction

In the US, there are approximately 300 rendering plants which produce various products including lard, grease, animal and marine protein meals and meat and bone meal generating an annual revenue of $2.8 billion (Economic Census-2002, US Census Bureau report). According to Swisher (2009), 8.7 billion chickens were slaughtered in US resulting in production of 2.4 billion pounds of poultry byproduct meal, 1.3 billion pounds of poultry fat and 1.2 billion pounds of feather meal.

Rendering is a process that "cooks" inedible by-products of the meat and poultry industry at high temperatures (115°C to 137°C). After cooking, fat is separated to produce lard, grease and tallow with the remaining by-product being pressed to produce protein meal. Poultry protein meal is defined as a dry rendered product from a combination of clean flesh and skin with or without accompanying bone, derived from parts of whole carcasses of poultry or combination thereof, exclusive of feathers, heads, feet and entrails (AAFCO, 2004).

Antioxidant activity of poultry meal is an index of its oxidative stability. With oxidation, the product will lose its sensory and nutrition quality attributes and may result in the formation of toxic compounds posing potential health concerns. Toxic compounds formed by oxidation reactions can lead to several diseases such as atherosclerosis, cytotoxicity, mutagenesis and carcinogenesis (Gray et al., 1996).

There are concerns about the use of synthetic antioxidants such as ethoxyquin, butylated hydroxy anisole (BHA), and butylated hydroxy toluene (BHT) in poultry protein meals due to the toxicity above certain limits (Branen, 1975; Ito, et al., 1986) .

Thus, there is a need to explore antioxidants naturally present in poultry protein meals, which could replace/minimize the use of synthetic antioxidants. In a recent article, Aldrich (2007) stated a need for natural water soluble antioxidants that could be applied in the processing environment.

Carnosine does not have any known toxic effects and excess carnosine is excreted in the urine (Perry et al., 1967). Furthermore, there are no reports of toxic effects of carnosine derivatives (Quinn et al., 1992; Sato et al., 2008). Carnosine is a water soluble dipeptide composed of β-alanine and L-histidine. It acts as a buffer (Davey, 1960; Skulachev, 2000; Smith, 1938) and antioxidant (Chan et al., 1994; Kohen et al., 1988) in muscle, aids in muscle contraction (Avena and Bowen, 1969; Severin et al., 1963) acts as a neurotransmitter in brain (Trombley et al., 2000; Tomonaga et al., 2004; Tomonaga et al., 2005), regulates calcium proteins in cardiac muscles (Roberts and Zaloga, 2000), possess anti-ageing effects (Hipkiss, 1998; Hipkiss and Brownson, 2000; Reddy et al., 2005) and chelates metal ions (Baran, 2000; Chan and Decker, 1994; Kohen et al., 1988). Carnosine was isolated from beef muscle in 1900 (Guelwitsch and Amiradgibi, 1900; Guelwitsch, 1906) and since then carnosine has been extracted and studied in a variety of animals. For example, carnosine was extracted from mammalian skeletal muscle of cat, dog, deer, gnu, oppossum and llama (Wolff and Wilson, 1935) and turkey (Davis et al., 1978). Carnosine is present as high as 20 mM in mammalian skeletal muscles and in lower levels in the central nervous system (Guiotto et. al., 2005). Carnosine has been extracted from mechanically deboned pork (Gopalakrishnan et al.,

1999), poultry breast (Maikhunthod et al., 2005) and from isolated muscle protein waste material (James et al., 1995).

Several researchers have reported various modes of antioxidant activity for carnosine. It acts as a metal chelator, reactive oxygen scavenger, free radical scavenger and peroxide decomposer (Baran, 2000; Chan and Decker, 1994; Reddy et al., 2005; Decker and Faraji, 1990). Very little information is available on the extraction of antioxidants such as carnosine from poultry meals. Therefore, the objective of the present research was to determine the concentration of carnosine from different poultry protein meals as well as to measure its antioxidant properties using different antioxidant methods.

4.2 Materials and Methods

4.2.1. Materials

Trolox- [(±)- 6-hydroxy-2, 5, 7, 8- tetramethyl-chroman-2-carboxylic acid; CAS# 53118-07-1], ferrous chloride [CAS# 7758-94-3], ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"- disulfonic acid sodium salt; CAS#69898-45-9], TCA [trichloroacetic acid; CAS# 76-03-9], DPPH [2,2-diphenyl-1-picryl-hydrazyl; CAS# 1898-66-4], fluorescein sodium salt [CAS# 518-47-8], 2-thiobarbituric acid [CAS# 504-17-6], L- carnosine [CAS # 305-84-0], OPA [phthaldialdehyde reagent; Product # 057K5015], L-α-phosphatidylcholine Type IV-S [CAS# 8002-43-5], AAPH [2,2'-

azobis (2-methylpropion-amidine) dihydrochloride 97% ; CAS # 2997-92-4] and TEP [3,3,3-tetarethoxypropane; CAS# 122-31-6] were purchased from Sigma Aldrich (St. Louis, Missouri, USA).

Sodium acetate trihydrate [CAS# 6131-90-4], methanol- HPLC grade (0.2µm filtered) [CAS # 67-56-1], acetonitrile- HPLC grade (0.2µm filtered) [CAS # 75-05-8] were purchased from Fisher Scientific (Fair-lawn, New Jersey, USA) while L-ascorbic acid sodium [CAS # 134-03-2 was purchased from Arcos Organics (New Jersey, USA) while BHT [butylated hydroxytoluene] CAS# 128-37-0 was purchased from MP Biomedical, Inc (Solon , Ohio, USA). All reagents were ACS grades or purer.

4.2.2. Sample Procurement

Pet grade poultry protein meal samples, sample-A and sample-G were obtained from two different rendering facilities without added stabilizers. Carnosine was extracted using a hot water procedure described by Maikhunthod et al., (2005) with slight modifications (Figure 4.1). Briefly, one part of ground poultry protein meal sample was added to six parts of pre-cooled (4°C) nano pure water and samples were homogenized in a Osterizer Blender (Model # 4937, Sunbeam Products Inc., Boca Raton, FL, USA) using 4 cylcles of 2min each cycle with 2 min cooling with ice slush between each cycle (8 min total homogenization). The homogenate was centrifuged at 20,000 g for 30 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA); the supernatant was then filtered through Whatman #4 filter paper (GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA). The water extracted filtrate

(supernatant) was subjected to heat treatment at 80°C for 15 min in a jacketed water bath (Precision, Model#283, Thermo scientific, Winchester, VA, USA), followed by cooling in ice water for 5 min. The heat extract was centrifuged to remove precipitated proteins at 6000 g for 20 min then the supernatant was filtered through Whatman #4 filter paper.

The collected supernatant was then ultrafiltered using Amicon Ultra-15 filter device (Catalog # UFC900324, Millipore, Billerica, MA, USA) with 3000 molecular weight cut off, using centrifugal force of 4000g for 55 min at 14°C in Beckman Model J-6B centrifuge (Beckman Coulter Inc., Brea, CA, USA). The ultrafiltration permeate was collected and stored at -80 °C until further analysis.

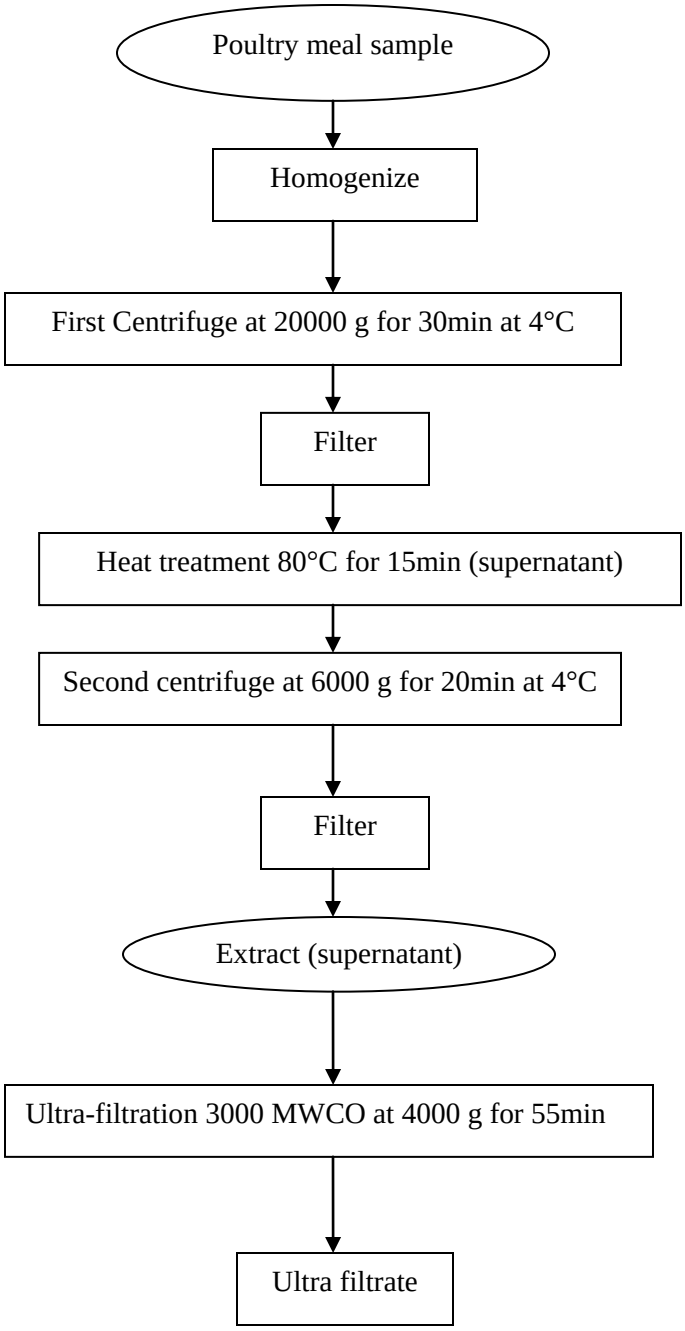

Figure 4.1: Flow Chart of extraction Procedure

4.2.3. Determination of carnosine by HPLC Method

Carnosine content was measured by HPLC as described by Gopalakrishnan et al., (1999) and Maikhunthod et al. (2005) with slight modifications. 2 ml of 0.4M perchloric acid was added to 2 ml of poultry protein meal ultrafiltrate. The mixture was vortexed (Vortex Genie 2 ^{TM}, Model# G-560, Fisher Scientific, Bohemia, New York) and boiled for 10 min (to precipitate proteins) and then centrifuged at 5000g for 5 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA). After centrifugation, the supernatant was filtered through 0.45 µm membrane filter. Filtrate was derivatized using OPA by adding 100 µl of OPA to 500 µl of sample, just prior to injection into HPLC (Shimadzu Instruments, Columbia, MD, USA).

OPA derivatized carnosine was separated using a mobile phase of 0.3M sodium acetate (pH 5.5), methanol and acetonitrile (75:15:10) at a flow rate of 0.75 ml/min with Waters Spherisorb SCX-4.6x250 mm column (Waters Corporation, Milford, MA, USA). Derivatized carnosine was detected using fluorescence (RF551 Spectroflurometric detector, Shimadzu Instruments) with an excitation wavelength of 310 nm and emission wavelength of 375 nm. The standard curve was prepared using a carnosine solution (5– 80mM). The retention time and peak areas were analyzed by EZ start 7.4 Software provided with the equipment (Shimadzu Instruments, Columbia, MD, USA).

4.2.4. TBARS Inhibition

TBARS inhibition was analyzed using a phosphatidyl choline emulsion system, as described by Kansci et al. (1997) and Gopalkrishnan et al. (1999). TBA/TCA reagent was prepared by a method described by Tarladgis et al. (1960). Two mg/ml phosphatidyl choline emulsion was prepared in a 5mM phosphate buffer pH 7.0 using Polytron® PT2100 homogenizer (Capitol Scientific Inc., Austin, Texas, USA). The phosphatidyl choline emulsion (1.8 ml) was mixed in 0.5 ml of sample and held at room temperature for 5 min to allow sample to react with the emulsion. After 5 min, the emulsion oxidation was initiated using catalyst $FeCl_2$ (50 µl) and sodium ascorbate (100 µl) to the final concentration of $FeCl_2$ and sodium ascorbate of 40µM each in emulsion system.

The polypropylene tubes (BD Falcon, Mississauga, ON, Canada) containing mixture were incubated at 37°C in a water bath (Precision, Model#283, Thermo scientific, Winchester, VA, USA) and after 2 hours the reaction was stopped by adding 50 µl of 10% BHT solution. To each tube, 2.5ml of TBA/TCA solution was added and vortexed with Vortex Genie 2 ^{TM} (Model# G-560, Fisher Scientific, Bohemia, New York) followed by heating in water bath at 90°C for 15 min. After heat treatment, the tubes were cooled down under running tap water and then centrifuged at 5000 g for 15 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA).

Absorbance was read at 531 nm using a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, New Jersey, USA) and water was used as a blank.

MDA (malondialdehyde) was calculated using standard curve prepared from TEP (1, 1, 3, 3,-tetraethpxypropane) at concentrations from 0 to 70 nM MDA. All reagents were prepared fresh daily prior to experimentation. Emulsion without extract was taken as a negative control.

Percent TBARS Inhibition was calculated using following formula:-

{(MDA without extract-MDA with extract) / MDA without extract} x100

4.2.5. Free radical scavenging assay

Free radical scavenging assay was performed as described by Yen et al. (2002). DPPH radical (0.2mM) was dissolved in absolute ethanol. 2ml of poultry protein meal ultrafiltare was mixed with 2ml of DPPH. The mixture was allowed to stand for 30 min in dark and the absorbance of the resultant solution was measured at 517nm with a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, New Jersey, USA). Mixture without extract was taken as negative control and absolute ethanol was used as a blank.

Percent free radical scavenging was calculated as {(Absorbance of control at 517 nm – Absorbance of sample at 517 nm)/Absorbance of control at 517 nm} x 100

4.2.6. Metal Chelating Activity

Chelating activity of Fe^{2+} was measured using a method described by Yen and Wu (1999). In this method, 1ml of poultry protein meal ultrafiltrate was added to 3.7 ml of nanopure water and the mixture was reacted with 100 µl of 2mM $FeCl_2$ and 200 µl of

5mM ferrozine for 20 min. After 20min absorbance was measured at 562 nm using a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, New Jersey, USA). Nanopure water was used as a blank. A negative control (sample with all reagents but no poultry meal ultrafiltrate) was used for calculations and the percent chelation was calculated using formula:-

{1- absorbance of the sample at 562 nm/ absorbance of control at 562 nm} x 100

4.2.7. ORAC Assay

Oxygen radical absorbing capacity (ORAC) was determined by a hydrophilic method described by Wu et al. (2008). The poultry protein meal samples were diluted 100 times with phosphate buffer (pH 7.4). Twenty five microliter of the diluted sample or Trolox standard solution (0, 10, 20, 40, 60,100µM) or blank (phosphate buffer pH 7.4) was added to one of 96 well plates (black with clear bottom, Optilux ^{TM}, BD Falcon). Flourescein solution (150µl) of 0.004 µM concentration was added to each well and the microplate was incubated at 37°C for 30 min in VWR-Incubating mini shaker (Model#980150, VWR, Arlington Heights, IL, USA). After 30 min incubation at 37°C, 25µl of AAPH solution (153mM) was added to each well (as the peroxyl generator) using auto injector (BioTek Instruments, Inc. Winnooski, Vermont, USA) to inititate the reaction. The microplate reader Synergy-HT from BioTek Instruments, Inc (Winnooski, Vermont, USA) was programmed to measure fluorescence at an excitation wavelength of 485 nm and an emission wavelength of 520nm at 1min time intervals for 1 hr and ORAC values were calculated using software Gen5^{TM} (BioTek Instruments, Inc. Winnooski,

Vermont, USA). The data were expressed as Trolox equivalents (TE) per gram of original sample (dry basis).

4.2.8. Total Mineral Content

Standard mineral content in a poultry protein meal was measured from ultrafiltrate samples diluted 1:10 before measurment. Samples were sent to the Agricultural Services Laboratory at Clemson University where mineral content was determined using inductively coupled plasma spectrometer (SPECTRO ARCOS- ICP, Kleve, Germany).

4.2.9. Protein Content

Poultry protein meal samples were sent to Agricultural Services Laboratory at Clemson University where protein content was determined with Dumas method described in the Official Methods of Analysis, section 968.06 (AOAC, 1990) using nitrogen/protein analyzer (LECO Model# FP 528 ,Warrendale, PA, USA) .

4.2.10. Statistical Analysis

ANOVA was performed to determine overall differences in the group means. To determine specific differences between pairs of group means, the Fisher LSD test was used. Both tests used a Type I Error probability of 0.05 and the SAS (Statistical Analysis Software Edition 9.2, SAS Institute Inc., 2007) was used to perform the statistical calculations.

4.3. Results and Discussion

4.3.1. Proximate composition and Carnosine Content

Sample-A showed higher ($p \leq 0.05$) moisture and protein content than sample-G (Table 4.1). Ash content of sample-A did not differ ($p \geq 0.05$) from sample-G. In addition, total mineral content of sample-A was higher than sample-G (Table 4.2).

Standard pet food grade contains less than 14% ash while low ash poultry meal or poultry by product meal contains 11% or less ash (Aldrich, 2007). In present study, ash content of poultry protein meal samples were greater than 11%, indicates that the samples were pet grade. Feed grade is seldom used in pet food because it contains lower levels of protein and higher levels of ash (Aldrich, 2007).

Carnosine content in poultry protein meal sample-G was nearly 2.6 times higher ($p \leq 0.05$) than that of sample–A (Table 4.1). Presence of carnosine in the meat and bone meal, fish meal and feather meal has been determined using HPLC (Schonherr 2002) whose primary objective was to develop a reliable and cost effective analytical method. This study revealed the carnosine presence in different animal origin feeds, and also indirectly hinted as to carnosine integrity after high temperature processing (~115°C). Carnosine content results in the current study were in agreement with Schonherr (2002).

Antioxidant Activity Analysis

There is no one standardized method to measure all aspects of antioxidant activity and for this reason different methods for measuring antioxidant activity under various oxidation conditions approach was employed (Di Bernardini et al., 2011; Zulueta et al.,

2009; Frankel and Meyer, 2000). The antioxidant activity of poultry protein meal ultrafiltrate was measured using thiobarbituric acid reactive species (TBARS) inhibition, ORAC, metal chelating and DPPH radical scavenging assay.

4.3.2. TBARS Inhibition

TBARS primarily measures the malondiadehyde (MDA) content, an abundant product of lipid oxidation as well as other secondary products of the oxidation reaction. TBARS inhibition of Sample-G (15.86%) was slightly greater than carnosine-10mM standard (11.09%); however this difference was not significant at the 5% level. Sample-A did not exhibit any TBARS inhibition (Table 4.3). Overall comparison of percent TBARS inhibition, free radical scavenging and metal chelation of poultry meal samples were compared in Figure 4.2.

Inhibition of TBARS by poultry protein meal in our study was lower than those reported by Maikhunthod and Intarapichet (2005) for broiler's breast and thigh ultrafiltrate. These differences in results between poultry protein meal and similar raw tissues may be due to source differences, concentrations of phosphatidyl choline emulsion in the system, and the variation in catalysts ($FeCl_2$ and sodium ascorbate) levels. Further, some enzymes which impart antioxidant activity similar to carnosine such as superoxide dismutase, catalase or glutathione oxidase and glutathione reductase (Sacchetti et al., 2008 and Chan et al., 1994) may be denatured or lose activity to different degrees during the high temperature rendering process. TBARS inhibitions

found in the current study were similar to values reported for mechanically separated pork extract (Gopalakrishnan et al., 1999).

Carnosine is known to form complexes with certain divalent metal ions such as Co, Cu, Mn, Zn, Cd, while K, Mg, Ca, Na do not bind with carnosine (Boldyrev, 2007). The metal ions already present (Co, Cu, Mn, Zn, Cd) in the extract might promote this reaction or act as proxidants (when added into the emulsion system of phosphatidyl choline) and thus, can lead to interference of the results. Poultry meal contains amino acids such as histidine, lysine, methionine, tryptophan, tyrosine and cystein (Aldrich, 2007), all of which have antioxidant properties.

Aromatic amino acids such as histidine, tyrosine and tryptophan can donate protons or electrons to scavenge radicals and stop the oxidation reaction while non-aromatic amino acids such as methionine, lysine and cysteine can interact directly with radicals (Sarmadi and Ismail, 2010). Other hydrophilic antioxidants such as small peptides like anserine, homocarnosine, uric acid and ascorbates may be present in the poultry meal (Sacchetti et al., 2008). Moreover, protein denaturation due to high temperature (~115°C), may cause changes in amino acid sequences of histidine and other amino acids such as lysine and proline to form new peptides (for example P-H-H or L-L-P-H-H) (Di Bernardini et al., 2011) which can provide antioxidant properties in poultry protein meal. Water soluble molecules (amino acids and other small peptides) are also extracted with carnosine. Thus, it is likely that these substances along with carnosine (present in extract) contribute to the antioxidant activity of poultry protein meal.

4.3.3. Metal Chelating Activity

Metal chelating activity of carnosine-10mM standard was higher ($p \leq 0.05$) than that of sample-A and sample- G (Table 4.3). Sample–A and sample-G metal chelating activity did not differ ($p \geq 0.05$).

Carnosine acts as a metal chelator and interrupts oxidation reactions (Baran, 2000). Carnosine forms complexes with Fe^{2+}. Amino acids such as histidine also form complexes with Fe^{2+} which can results in a mixed ligand formation (Boldyrev, 2007) and can alter metal chelation values.

Therefore, as suggested by various scientists, the removal of proxidants such as iron will increase antioxidant activity of protein extracts (Chan et al., 1993; Gopalakrishnan et al., 1999; Maikhunthod and Intarapichet, 2005). So, for a more accurate evaluation of the antioxidant activity of carnosine in poultry meal, demineralization could be an option. However, for commercialization the addition of demineralization step would add cost to the final product.

4.3.4. Free Radical Scavenging (DPPH)

DPPH assay is based on electron donation by antioxidants to stabilize the electron deficient DPPH radical. Free radical scavenging activity of sample-G and sample-A was greater ($p \leq 0.05$) than that of the carnosine-10mM standard, while sample-A and sample-G did not differ ($p \geq 0.05$) (Table 4.3).

Besides carnosine, aromatic amino acids (such as histidine, tyrosine and tryptophan) can donate proton or electron to deficient radicals and also interrupt the

oxidation reaction. Cysteine, a non-aromatic amino acid can directly interact with radicals (Sarmadi and Ismail, 2010) while polyamines also contribute radical scavenging activity (Sacchetti et al., 2008) . Thus, carnosine is not only the component in protein meal that can impact free radical scavenging activity.

4.3.5. ORAC Values

ORAC assay is based on hydrogen atom donation by antioxidants to stabilize peroxyl radicals produced by AAPH. In this method, the ORAC values are obtained from kinetic curves derived from competitive kinetic reactions (Huang et al., 2005). Sample-A ORAC values (μM Trolox Equivalents/gm of dry sample) were higher ($p \leq 0.05$) than those of sample-G (Table 4.3).

ORAC values of poultry protein meal ultrafiltrates were higher than those of hydrophilic extract of beef samples obtained by Wu et al., (2008). Kohen et al., (1988) determined antioxidant activity of carnosine, homocarnosine and anserine against peroxyl radicals by using voltametric measurements in AMVN {2,2-azobis (2,4-dimethylvaleronitrile)} and AAPH {2,2'-azobis(2-amidino-propane dihydrochloride)} systems under physiological conditions. They also compared different structures such as carnosine, anserine, GABA {γ-amino butyric acid}, L-alanine, β-alanine and found that carnosine has 53% inhibition against peroxyl radicals while anserine showed 39% and GABA, L-alanine, β-alanine showed no inhibition. Histidine displayed 42% while histamine showed 28% inhibition against peroxyl radicals due to donation of hydrogen

atom to the peroxyl radicals. Our samples might contain other dipeptides such as anserine, homocarnosine which interact with peroxyl radicals in similar way as carnosine. Several researchers have reported various modes of antioxidant activity for carnosine. It acts as a metal chelator, reactive oxygen scavenger, free radical scavenger and peroxide decomposer (Baran, 2000; Chan and Decker, 1994; Reddy et al., 2005). Carnosine also possesses antioxidant properties due to the imidazole moiety in its structure (Aruoma et al., 1989; Boldyrev et al., 1993; Boldyrev et al., 1997; Hartman et al., 1990; Kohen et al., 1988; Quinn et al., 1992)

From the current study, and one by Kansci et al., (1997) it can be concluded that carnosine antioxidant activity is multifunctional; It chelates metal ions, decreases free radicals and reacts with secondary oxidation products.

4.4. Conclusions

Besides antioxidant properties, carnosine has other potential therapeautic as well as medicinal values. Carnosine exhibits anti-ageing effect (Hipkiss, 1998; Hipkiss and Brownson, 2000; Reddy et al., 2005), reduces physiological oxidative stress (Boldyrev, 2007), aids wound healing and inhibits inflammation (Boldyrev and Severin, 1990; Nagai, 1980). Chelating properties of carnosine against metal ions (Baran, 2000; Chan and Decker, 1994; Kohen et al., 1988) can be exploited to cure Wilson's disease (by chelation of Cu ions). Carnosine is a potential cure for senile cataracts (Babizhayev et al., 2009) and Alzheimer's disease (Reddy et al., 2005). Buffering properties of

carnosine in muscle (Davey, 1960; Skulachev, 2000; Smith, 1938) improve high intensity exercise performance and endurance in humans (Sato et al., 2003). Carnosine zinc complex (L-CAZ, generic name Polaprezinc) is the first drug administered orally for anti-ulcer effect due to its membrane protection ability (Matsukura and Tanaka, 2000).

Carnosine extraction from poultry meal using hot water would have a relatively low manufacturing cost having excellent solubility in pet food and easy digestion in animals. Moreover, there is an increasing demand of producing novel functional foods containing bioactive peptides such as carnosine, anserine and L-carnitine (Arihara, 2006). Therefore, it can be concluded that extraction of carnosine from poultry meal may have a positive economical impact by increasing revenue for the rendering industry. By carnosine incorporation, potential therapeutic pet food could be exploited to benefit animal health.

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TABLES and FIGURES

Table 4.1: Proximate Composition of Poultry Protein Meals

Sample	Moisture Content (%)	Protein Content (wb) [1] (%)	Protein content (db) (%)	Ash Content (%)	Carnosine Content (wb) [1, 3]	Carnosine Content (db)
Sample-A	4.96±0.06 [a]	67.6±1.48 [a]	70.1±1.48 [a]	13.28±0.16[a]	38.28±0.46 [a]	40.28±0.49[a]
Sample-G	2.16± 0.07 [b]	65.4±2.03 [b]	69.4±2.03[a]	12.76±0.20[a]	102.44±10.06[b]	104.71±10.28[b]

1. HPLC method was used for determination of carnosine content; carnosine content is expressed in mg/ 100 gm of original sample. Wb= wet basis and db= dry basis.
2: All values are Mean ± SEM (N=3)
3: Fisher's Least Significant Difference Test was used to compare mean values; [a-b] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).

Table 4.2: Mineral Composition of Poultry Protein Meals [1]

Sample	Mineral Composition											
	P	**K**	**Ca**	**Mg**	**Zn**	**Cu**	**Mn**	**Fe**	**S**	**Na**	**B**	**Al**
Sample-A	546.29	1686.21	62.32	71.85	0.22	0.05	0.03	0.66	292.34	769.26	0.24	0.02
Sample-G	398.14	1247.28	42.28	47.85	0.08	0.10	0.02	0.55	221.67	751.99	0.16	0.18

1: Concentrations are parts per million (ppm) on dry basis.

Table 4.3: Antioxidant Activity Tests

Sample	Antioxidant Activity Test			
	TBARS Inhibition (%) [1]	**Metal Chelation (%) [2]**	**Free Radical Scavenging (%) [3]**	**ORAC Values [4]**
Sample-A	No activity detected	64.16±5.12[a]	81.41±0.19[a]	84.35±0.34[a]
Sample-G	15.86±2.01 [a]	63.78±4.53[a]	84.17±0.50 [a]	68.44±1.36[b]
Carnosine-10Mm	11.09±0.98 [a]	94.93±1.65[b]	8.93±1.75 [b]	Not Determined

1) All values are Mean ± SEM (N=4); ND= Not determined
2) a, b means within columns with different superscripts are significantly different ($p \leq 0.05$)
3) % TBARS Inhibition= {(MDA without extract-MDA with extract) / MDA without extract} x100
4) % Metal chelating activity= {1- absorbance of the sample at 562nm/ absorbance of control at 562nm} x 100
5) % Free radical scavenging was calculated as {(Absorbance of control at 517 – Absorbance of sample at 517)/Absorbance of control at 517} x 100
6) ORAC Assay (Oxygen Radical Absorbing Capacity); Values are expressed in Trolox equivalents (TE) per gram of original sample (dry basis).
7) Fisher's Least Significant Difference Test was used to compare mean values; [a-b] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).
8) Carnosine was used as standard for comparisons.

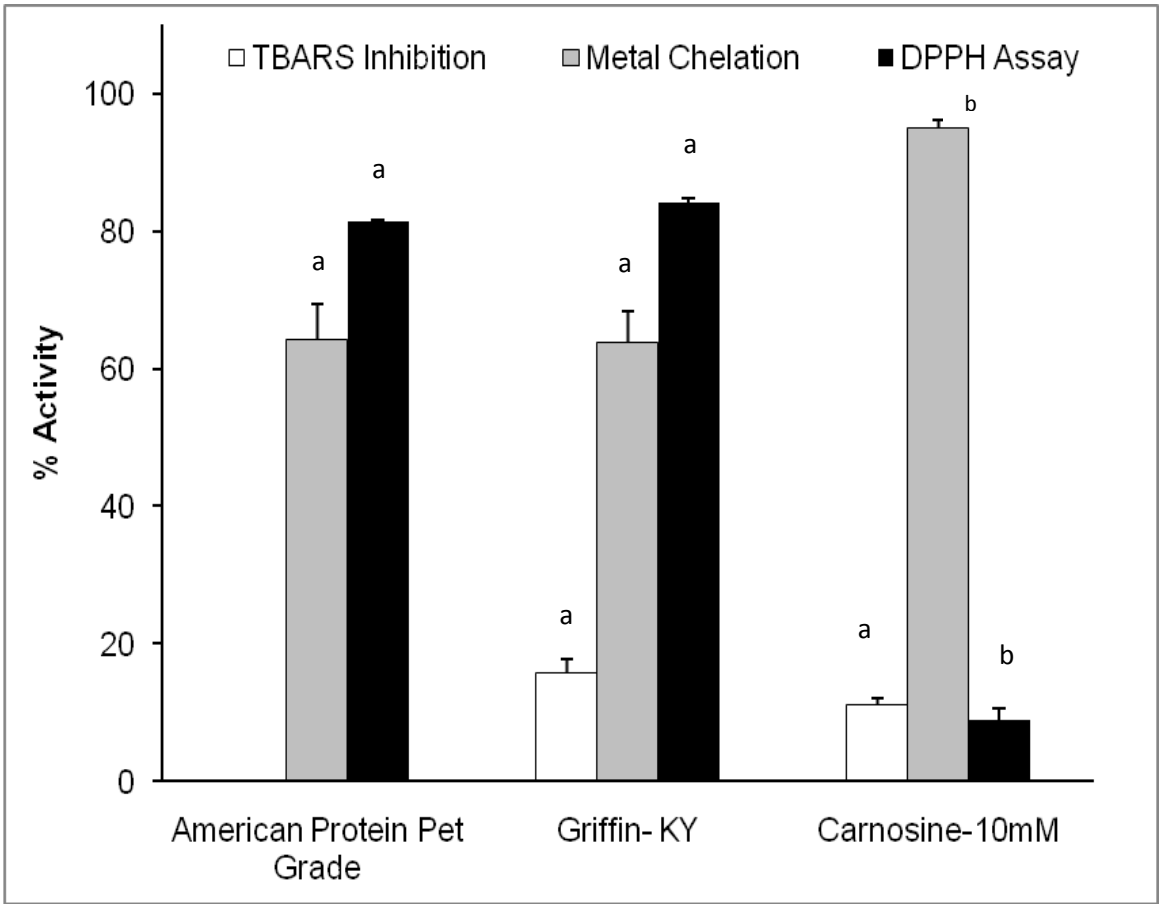


Figure 4.2: Antioxidant activities of poultry meal using different antioxidant methods. Carnosine was used as standard for comparison. Different letters indicate that the values are significantly different ($p \leq 0.05$).

CHAPTER FIVE

ANTIOXIDANT ACTIVITIES OF CARNOSINE AND ITS CONSTITUENT AMINO ACIDS IN DIFFERENT MODEL SYSTEMS

Abstract

The present study compared carnosine with its constituent amino acids, β-alanine and L-histidine as well as with imidazole to elucidate the antioxidant mechanism of carnosine and its components. Concentrations ranging from 5 to 100 mM of carnosine, L-histidine, β-alanine and imidazole were prepared and antioxidant activity assays (TBARS inhibition, metal chelating activity, free radical scavenging activity) were conducted.

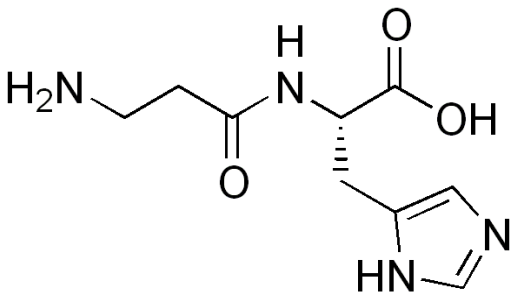
Our results indicate that TBARS inhibition of carnosine was due to the imidazole ring present in the histidine and with no inhibition contributed to β-alanine. Metal chelating properties of carnosine was also due to the imidazole ring and not to histidine or β-alanine, while free radical scavenging activity of carnosine was attributed to histidine amino acid and not due to imidazole and β-alanine.

Overall, results indicate that β-alanine and the peptide bond present between L-histidine and β-alanine do not play any role in antioxidant activity of carnosine. Furthermore, we determined that the imidazole has antioxidant properties alone, therefore, could be used as an antioxidant in various food and feed applications.

5.1 Introduction

Carnosine is a water soluble dipeptide composed of β-alanine and histidine that includes imidazole (Figure 1a, 1b, 1c, 1d). Histidine is an essential amino acid for humans while β-alanine is non-essential amino acid being synthesized in liver as a final product of uracil and thymine degradation (Matthews and Traut, 1987). Carnosine acts as a buffer in muscle tissue (Davey, 1960; Skulachev, 2000; Smith, 1938), a potent antioxidant in skeletal muscles (Chan and Decker, 1994; Kohen et al., 1988), aids in muscle contraction (Avena and Bowen, 1969; Severin et al., 1963) and acts as a neurotransmitter in brain (Trombley et. al, 2000; Tomonaga et al., 2004; Tomonaga et al., 2005). Futhermore, carnosine is a regulator of calcium proteins in cardiac muscles (Roberts and Zaloga, 2000), possesses anti-ageing effect (Hipkiss, 1998; Hipkiss and Brownson, 2000; Reddy et al., 2005) and a chelating agent of metal ions (Baran, 2000; Chan and Decker., 1994; Kohen et al., 1988). Various mechanisms of carnosine antioxidant activity have been reported, including metal chelation, reactive oxygen scavenging, free radical scavenging and peroxide decomposing (Baran, 2000; Chan and Decker., 1994; Kohen et al., 1988). The structure-function relationships of carnosine indicates that the imidazole moiety of carnosine and the peptide linkage between histidine and β-alanine are responsible for its antioxidant activity (Aruoma et al.,1989; Boldyrev et al., 1993; Boldyrev et al., 1997; Hartman et al., 1990; Kohen et al., 1988; Quinn et al., 1992).

The present study compared carnosine with its constituent amino acids, β-alanine and L-histidine as well as with imidazole to elucidate the antioxidant mechanisms of carnosine and its constituent amino acids.



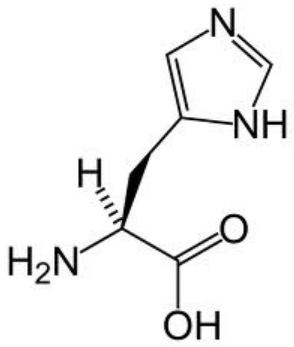
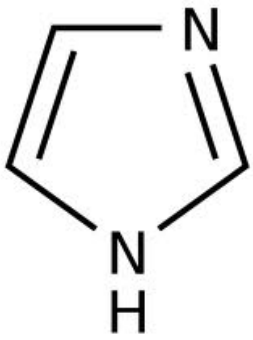
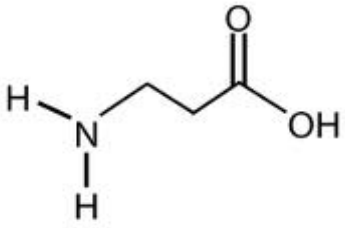


Figure5.1a: Two dimensional structure of carnosine 5.1 b: Two dimensional structure of histidine





5.1c: Two dimensional structure of imidazole 5.1d: Two dimensional structure of β-alanine

5.2. Materials and Methods

5.2.1. Materials

Ferrous chloride [CAS# 7758-94-3], ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-4',4"- disulfonic acid sodium salt; CAS#69898-45-9], TCA [trichloroacetic acid; CAS# 76-03-9], DPPH [2,2-diphenyl-1-picryl-hydrazyl; CAS# 1898-66-4], 2-thiobarbituric acid [CAS# 504-17-6], L- carnosine [CAS # 305-84-0], L-histidine [CAS# 71-00-1], β-alanine [CAS# 203-536-5] Imidazole [CAS#288-32-4], L-α-phosphatidylcholine Type IV-S [CAS# 8002-43-5], and TEP [3,3,3-tetarethoxypropane; CAS# 122-31-6] were all purchased from Sigma Aldrich (Saint Louis, Missouri, USA). L-ascorbic acid sodium [CAS # 134-03-2] was purchased from Arcos Organics (New Jersey, USA) while BHT [butylated hydroxytoluene; CAS# 128-37-0] was purchased from MP Biomedical, Inc (Solon, Ohio, USA). All reagents were ACS grades or purer.

Methods

5.2.2. Preparation of reagents

Samples of different molar concentrations (5, 25, 50, 100 mM) of L- carnosine, L-histidine, β-alanine and Imidazole were prepared using nano pure water just prior to experimentation. All reagents were prepared fresh daily.

5.2.3. TBARS Inhibition

Thiobarbituric acid reactive species (TBARS) inhibition was analyzed using a phosphatidyl choline emulsion system, as described by Kansci et al (1997) and

Gopalkrishnan et al (1999). TBA/TCA reagent was prepared by a method described by Tarladgis et al. (1960). Two mg/ml phosphatidyl choline emulsion was prepared in 5mM phosphate buffer (pH 7.0) using Polytron® PT2100 homogenizer (Capitol Scientific Inc., Austin, Texas, USA). 1.8 ml phosphatidyl choline emulsion of emulsion was mixed in 0.5 ml of sample and held for 5 min to allow interaction of carnosine or each of the constituents (i.e. histidine, β-alanine and imidazole) with the emulsion. After 5 min, the emulsion oxidation was intiated by adding catalyst $FeCl_2$ (50 µl) and sodium ascorbate (100 µl) achieving a 40µM concentration of $FeCl_2$ and sodium ascorbate in emulsion system.

Polypropylene tubes (BD Falcon, Mississauga, ON, Canada) containing the emulsion were incubated at 37°C in a water bath (Precision, Model#283, Thermo scientific, Winchester, VA, USA) and after 2 hours the reaction was terminated by adding 50 µl of 10% BHT solution. To each tube, 2.5ml of TBA/TCA solution was added and vortexed with Vortex Genie 2 ^{TM} (Model# G-560, Fisher Scientific, Bohemia, New York) followed by heating in water bath at 90°C for 15 min. After heat treatment, the tubes were cooled under running tap water and then centrifuged at 5000g for 15 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA).

Absorbance was read at 531nm using a spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, New Jersey, USA) and water was used as a blank. Malondialdehyde (MDA) was calculated using a standard curve prepared from 1, 1, 3, 3,-tetraethpxypropane (TEP) at concentrations from 0 to 70 nM MDA. All reagents were

prepared fresh daily prior to experimentation. Tubes containing reagents but without sample were taken as a negative control.

Percent TBARS Inhibition was calculated using following formula:-

{(MDA without extract-MDA with extract) / MDA without extract} x100

5.2.4. Free radical scavenging assay

Free radical scavenging assay was performed as described by Yen et al. (2002). DPPH radical (0.2mM) was dissolved in absolute ethanol. Two ml of carnosine or histidine or β-alanine or imidazole was mixed with 2ml of DPPH and the mixture was allowed to stand for 30 min in dark and the absorbance of the resultant solution was measured at 517nm with spectrophotometer (Genesys 20, Model #4001/4; Themofisher Scientific, New Jersey, USA). Mixture without sample was taken as negative control and absolute ethanol was used as a blank.

Percent free radical scavenging activity was calculated as {(Absorbance of control at 517 nm – Absorbance of sample at 517 nm)/Absorbance of control at 517 nm} x 100

5.2.5. Metal Chelating Activity

Chelating activity on Fe^{2+} was measured using the method described by Yen and Wu (1999). In this method, 1ml of carnosine or histidine or β-alanine or imidazole was added to 3.7ml of nanopure water and the mixture was reacted with 100 µl of 2mM $FeCl_2$ and 200 µl of 5mM ferrozine for 20 min kept at ambient temperature. After 20min absorbance was measured at 562nm using spectrophotometer (Genesys 20, Model

#4001/4; Themofisher Scientific, New Jersey, USA). Nanopure water was used as a blank. Tubes with no sample was used as a negative control

Percent chelation was calculated using formula:-

{1- absorbance of the sample at 562nm/ absorbance of control at 562nm} x 100

5.2.6. Statistical Analysis

ANOVA was performed to determine overall differences in the group means. To determine specific differences between pairs of group means, the Fisher LSD test was used. Both tests used a Type I Error probability of 0.05 and the SAS (Statistical Analysis Software Edition 9.2, SAS Institute Inc., 2007) was used to perform the statistical calculations.

5.3. Results and Discussion

5.3.1. TBARS inhibition

TBARS measures MDA content which is the most abundant product of lipid oxidation and other secondary products of reaction. Carnosine, histidine and imidazole showed a positive trend of increased inhibition with an increase in molar concentration of each constituent (Table 5.1). Imidazole displayed the greatest inhibition followed by histidine and carnosine ($p \leq 0.05$) at each molar concentration. L-histidine showed higher TBARS inhibition values than carnosine at 50 and 100mM ($p \leq 0.05$) while lower TBARS values than carnosine at 25mM ($p \leq 0.05$); the difference between carnosine and L-

histidine at 5mM concentration was not significant ($p \geq 0.05$). β-alanine did not exhibit TBARS inhibition at any molar concentrations tested. Results indicated that TBARS inhibition of carnosine was due to the imidazole ring present in the histidine. Erickson and Hultin also found that histidine inhibits lipid peroxidation in a system catalyzed by ferrous and ascorbate (Erickson and Hultin, 1992) .

5.3.2. Metal Chelating Activity

Metal chelating activity of carnosine was similar to imidazole with no significant differences ($p \geq 0.05$) at any concentrations tested (Table 5.1). Histidine did not exhibit metal chelating activity while β-alanine exhibited detectable metal chelation at all concentrations, however, these levels did not differ between levels ($p \geq 0.05$). The data collected revealed that carnosine exhibited metal chelation because of its imadazole ring and not due to histidine, β-alanine or the presence of a peptide bond.

Carnosine acts as a metal chelator to retard oxidation reactions forming complexes with copper ions, zinc, vanadium, Ni II and Mn II ions (Baran, 2000). The protonated nitrogen (N^3) in the imidazole ring interacts with Cu II ions and Zinc (Zn II) and thus makes a stable metal complex (Chan and Decker., 1994), but it does not form a complex with iron (Fe III) (Decker et al., 1992). However, the results of the current study verify those of Kohen et al, (1988) that carnosine forms a complex with Fe^{2+} in the same manner it does Cu^{2+}. Thus, carnosine might stabilize feedstuffs against oxidation by chelating Fe^{2+} and also as a carrier in diet to facilitate absorption.

5.3.3. Free Radical Scavenging Activity

Free radical scavenging activity increased with concentration for carnosine and histidine ($p \leq 0.05$) (Table 5.1), while β-alanine and imidazole showed slight to no scavenging of free radicals ($p \geq 0.05$). Carnosine exhibited free radical scavenging activity due to histidine and not due to imidazole ring present in its structure. These results are in agreement with Wu et al. (2003). Kohen et al (1988) suggested that the hydrogen's on the methylene carbon next to the imidazole ring are the likely proton donor terminating the oxidation reaction (Kohen et al, 1988) caused by free radicals. In our study, data for free radical scavenging activity of histidine (at 25mM exhibited 4.48 % scavenging) was slightly lower than Wu et al (2003) (at 20mM exhibited 7.4% scavenging). This difference was likely due to lower concentration of DPPH (0.1mM) used by these authors compared to present study.

Oxygen radical scavenging capacity of carnosine, homocarnosine and anserine against peroxyl radicals has been studied by Kohen et al. (1988) by using voltametric measurements in AMVN {2, 2-azobis (2, 4-dimethylvaleronitrile)} and AAPH {2, 2'-azobis (2-amidino-propane dihydrochloride)} systems under physiological conditions. They also compared different structures such as carnosine, anserine, GABA {γ-amino butyric acid}, imidazole, L-alanine, β-alanine and found that inhibition of peroxyl radicals by carnosine was 53%, histidine 42%, imidazole 39%, and anserine 39% while GABA, L-alanine and β-alanine showed no inhibition. Inhibition against peroxyl radicals is due to donation of a hydrogen atom to peroxyl radicals. Carnosine, histidine and β-

alanine results from Kohen et al (1988) were similar to the current study while those for imidazole differed. The un-availability of electrons in the imidazole ring to donate to the DPPH radical in the current study verses the availability of hydrogen atom to scavenge peroxyl radicals used by Kohen et al (1988) may be the reason for differing results for the imidazole.

Wu et al (2003) measured antioxidant activity of carnosine, anserine, histidine, β-alanine, 1-methyl histidine and their combinations at different concentrations using a ferric thiocyanate method to measure lipid autoxidation, DPPH radical scavenging assay and Cu^{2+} chelating ability. They concluded that the peptide linkage in carnosine is involved in its antioxidant activity. Similar conclusions were drawn by Chen and Decker (1994) and supported that the antioxidant properties of carnosine could also be attributed to the peptide bond present between β-alanine and histidine and not to histidine and β-alanine alone. Similar conclusions were also drawn by Yen et al (2002).

5.4. Conclusions:

Based on the present study, the peptide bond may not contribute significantly to antioxidant activity since the carnosine, histidine and imidazole exhibited antioxidant ability in all tests while β-alanine didn't show strong activity in any test conducted. Therefore, it can be concluded that histidine and imidazole ring of histidine are important for activity and not the peptide bond. Furthermore, these results were supported by

Kansci et al., (1997) who found that carnosine antioxidant activity is multifunctional and it chelates metal ions, decreases free radicals and reacts with some secondary oxidation products.

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Table 5.1: Antioxidant activities of carnosine and its constituents in different analysis.

	Conc.	Carnosine	Histidine	β-alanine	Imidazole
TBARS Inhibition (%)	**100mM**	12.83 ± 1.15 [e, f]	18.80 ± 0.48 [c]	0 ± 0.00 [i]	32.84 ±1.14 [a]
	50mM	11.21 ± 0.43 [g, f]	14.70 ± 1.58 [d, e]	0± 0.00 [i]	24.53±2.04 [b]
	25mM	8.32 ± 0.92 [g]	4.48± 0.41 [h]	0.05± 0.02 [i]	16.28± 1.50 [c, d]
	5mM	0.42 ± 0.41 [i]	0± 0.00 [i]	0 ± 0.00 [i]	4.62 ± 0.92 [h]
Metal Chelating Activity (%)	**100mM**	96.72 ± 1.31 [a, b]	0 ± 0.00 [d]	0.36 ±0.11 [d]	98.83 ± 0.37 [a]
	50mM	96.44 ± 1.54 [a, b]	0 ± 0.00 [d]	0.58 ± .20 [d]	98.94 ± 0.40 [a]
	25mM	92.74 ± 3.38 [a, b]	0 ± 0.00 [d]	0.82 ±0.21 [d]	98.79 ± 0.37 [a]
	5mM	86.56 ± 5.99 [b]	0 ± 0.00 [d]	0.83 ± 0.38 [d]	96.92 ± 0.97 [a, b]
Free Radical Scavenging Activity (%)	**100mM**	41.13 ± 12.40 [a]	18.44 ± 0.38 [c]	0.95±0.45 [h]	0.38± 0.14 [h, g]
	50mM	25.16 ± 7.58 [b]	13.51 ± 0.23 [d]	1.00±0.46 [h]	0.25± 0.09 [h]
	25mM	11.75 ± 3.54 [d]	10.42 ± 0.23 [e]	0.59 ± 0.20 [h, g]	0± 0.00 [h]
	5mM	1.86 ± 0.58 [g]	5.90 ± 0.22 [f]	0.71 ± 0.29 [h]	0 ± 0.00 [h]

1) TBARS Method; % Inhibition= {(MDA without extract-MDA with extract) / MDA without extract} x100
2) % Metal chelating activity= {1- absorbance of the sample at 562nm/ absorbance of control at 562nm} x 100
3) DPPH Assay; % Free radical scavenging = {(Absorbance of control– Absorbance of sample)/Absorbance of control } x 100
4) All values are Mean ± SEM (N=4); [a, b] values indicate within the column are significantly different ($p \leq 0.05$) while [a-i] similar letters indicate that the means values are not significantly different ($p \geq 0.05$).

CHAPTER SIX

EFFECT OF STRESS ON CARNOSINE LEVELS IN BRAIN, BREAST AND THIGH OF BROILERS

Abstract

The objective of the present study was to measure carnosine levels in different tissues of broilers under stress versus non-stress conditions. Heterophil-lymphocyte ratio and corticosterone levels were measured as indicators of stress. Corticosterone levels of stressed broilers (24358.67 pg/ml) were 10 folds higher (p=0.002) than non-stressed broilers (2275.46 pg/ml). However, there was no difference (p=0.29) in heterophil/lymphocyte ratio of non-stressed (0.29) and stressed birds (0.31). Carnosine content in breast of stressed birds (17.39 mg/gm) was 10 times higher (p=0.005) than non-stressed birds (1.85 mg/gm). Carnosine content in thigh of stressed birds (21.25 mg/gm) was approximately 2 fold higher (p=0.001) than non-stressed birds (11.10 mg/gm). Carnosine content in brain of stressed birds did not differ (p=0.82) from that in non-stressed birds. Based on the present study, carnosine may play significant role in muscle function during short term stress.

Abbreviations: H/L ratio - Heterophil-Lymphocyte ratio

Keywords: Carnosine, Stress, Corticosterone, broilers

6.1. Introduction

Broilers face a multitude of stresses during feed restriction, catching, handling and crating (Kannan and Mench, 1996; Nicol and Scott, 1990; Knowles and Broom, 1990), transportation (Yue et al., 2010), shackling (Bedanova et al., 2007) and exposure to heat (Altan et al., 2000) or cold (Campo et al., 2008; Maxwell, 1993) during housing or transportation.

Numerous studies have been conducted elucidating the effects of stress on the internal and external responses of chickens. The majority of research has indicated that stress negatively affects meat quality. Transportation stress reduces the meat quality (Pérez et al., 2002), reduces live weight (Kannan et al., 2000), reduces carcass weight and nutritional quality (Tankson et al., 2001). Long term transport causes increase in breast drip loss and affects the meat color (Yue et al., 2010) and results in smaller area and higher density of type II fibers (Zhang et al., 2009). Pre-slaughter stress reduces initial breast muscle pH, accelerates rigor mortis, decreases water holding capacity, increases paleness (Northcutt et al., 1994; Tankson et al., 2001), and increases protein hydrolysis (Dong et al., 2007). Thus, stress on chickens has been a major concern to the poultry industry as stress can potentially lower the meat quality as well as reduce the customer appeal.

There are multiple ways to measure stress in poultry including counting or examining heterophil-lymphocyte ratio (H/L ratio) and measuring corticosterone levels (Gross and Siegel, 1983). During stress, the levels of corticosterone increases, similarly

heterophil lymphocyte ratio, which is 0.4 normally in broilers (Siegel and Gross, 2000) increases with elevated stress in broilers (Gross and Siegel, 1983).

Carnosine is a natural water soluble dipeptide discovered from beef muscle in 1900 (Guelwitsch and Amiradgibi, 1900; Guelwitsch, 1906). On systematic analysis, it was found that this dipeptide is composed of β-alanine and histidine. Out of these two amino acids, histidine is an essential amino acid while β-alanine is a non-essential amino acid synthesized in liver as a final product of uracil and thymine degradation (Matthews and Traut, 1987).

Since its initial discovery, carnosine has been extracted from variety of animal species and from different tissues in order to better understand the significance of carnosine bodily functions. These studies have revealed that carnosine acts as neurotransmitter in brain (Trombley et al., 2000; Tomonaga et al., 2004; Tomonaga et al., 2005), as a buffer in the skeletal muscle (Davey, 1960; Skulachev, 2000; Smith, 1938), a potent antioxidant in skeletal muscles(Chan and Decker, 1994; Kohen et al., 1988) and aids in muscle contraction (Avena and Bowen, 1969; Severin et al., 1963). It also regulates calcium proteins in cardiac muscles (Roberts and Zaloga, 2000), exhibits anti-ageing effect (Hipkiss, 1998; Hipkiss and Brownson, 2000; Reddy et al., 2005) and acts as a chelating agent of metal ions (Baran, 2000; Chan and Decker, 1994; Kohen et al., 1988).

It has been reported in the previous studies that injection of carnosine into the brain of chicks induces hyperactivity and results in increase in blood plasma corticosterone levels (Tomonaga et al., 2004; Tsuneyoshi et al., 2007; Tsuneyoshi et al.,

2008). These studies found a relationship between carnosine and corticosterones and that during stress conditions the levels of corticosterone increases in birds indicating that carnosine may be a mitigating response to stress. Therefore, the objective of the present study was to measure carnosine levels in different tissues of broilers under short term stress versus non-stress conditions.

6.2. Materials and Methods

6.2.1. Materials

Sodium acetate trihydrate CAS# 6131-90-4, methanol- HPLC grade (0.2µm filtered) CAS # 67-56-1, acetonitrile- HPLC grade (0.2µm filtered) [CAS # 75-05-8] and ethyl acetate- CAS # 141-78-6 and EDTA-coated blood collection tubes (BD Vacutainer, Lot # 9029222) were purchased from Fisher Scientific (Fair-lawn, New Jersey, USA). OPA [phthaldialdehyde reagent; Product # 057K5015], L- carnosine [CAS # 305-84-0] was purchased from Sigma Aldrich (Saint Louis, Missouri, USA). Macneal stain- [Tetrachrome Stain- MacNeal; Catalog # 02783] was purchased from Polysciences. Inc (Warrington, Pennsylvania, USA) while Corticosterone Enzyme Immunoassay Kit- Catalog#900-097 was purchased from Enzo Life Sciences (Plymouth Meeting, Pennsylvania, USA).

6.2.2. Treatment of birds

The total number of birds needed to perform the study was 20 and was determined by using Power analysis program in SAS (Statistical Analytical Software, Edition 9.2) at a 90% power level. Twenty (6-7 weeks old) birds were collected from different pens (10 for stressed and 10 for non-stressed treatment). For the stressed treatment, the birds were held in the coops and driven in the back of a pick-up truck under sunny conditions for 15 min, followed by holding upside-down and wing flapping just before sacrificing. For the non-stressed treatment group, birds were acclimatimated to handling by lifting and holding them in the arms for approximately 3 min before sacrificing them. All birds were sacrificed or euthanized by cutting the jugular vein and bled to death (this method was used because the use of pentobarbital might potentially interfere with carnosine levels). These procedures were conducted under the supervision of a animal use official.

6.2.3. Sample collection and Preparation

Blood samples were collected from the jugular vein immediately after decapitation in EDTA-coated tubes and immediately stored in ice. After transport to the lab (~2 hours), plasma was separated from the blood by centrifugation for 15 min at 5000 g in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA) and supernatant (blood plasma) was collected in clean plastic tubes, which were stored at -80°C until analysis.

For heterophil - lymphocyte ratio, blood smears were made from the collected blood at the time of sacrificing and air dried at the collection site.

Breast, thigh and head samples were collected at the slaughtering site and kept in ice during transport to the laboratory (~2 hours). Upon return to the laboratory, the brain was removed from the head and stored under refrigeration (4±2°C).

6.2.4. Heterophill lymphocyte Ratio

Heterophill-lymphocyte ratios were determined by the staining method described by Conn (1940) and George (1981), and cells were viewed using informative notes provided by Glenn (2007). Smears were made by streaking a drop of blood over a clean glass slide using a second slide or a cover glass. Streaked slides were air-dried quickly at room temperature. Care was taken that the smears must be thin, preferably only one cell thick. The prepared slides were stained by placing 1 ml of MacNeal's Tetrachrome Stain (B-152-1) on the smears for 1-3 min, followed by adding 2ml of phosphate buffer (pH 7.0) and keeping the slides for 5-6 min. After 5-6 min, the stains were flooded and washed with phosphate buffer (pH 7.0) until thin portions of the smears were pink. The smears were dry blotted carefully and after fixing with one drop of cytoseal-60, smears were covered with cover slip and then observed under oil immersion lens (total magnification 1000) (Carl Zeiss Microimaging GmbH, Munich, Germany).

6.2.5. Corticosterone Assay

The corticosterone levels were determined using corticosterone enzyme immunoassay kit (Enzo Life Sciences catalog#900-097). Corticosterone from plasma was extracted in chemical fume hood by adding 1ml ethyl acetate to every ml of sample. A

total of three separate tubes for stress or non stress groups were made. The tubes were vortexed and kept for ~ 45min and the upper organic layer was removed three times and collected together in one tube. The collected organic layer was dried using gaseous nitrogen (to remove ethyl acetate) and tubes were stored at -80°C until analysis.

The dried samples were reconstituted with 500µl assay buffer provided in the enzyme immunoassay kit (Enzo Life Sciences catalog#900-097). Hundred microliter of corticosterone standards (32, 160, 800, 4000, 20000 pg/ml) were added to the standard wells while 100 µl samples were added to the sample wells and the protocol provided with the manufacturers kit was followed. The 96-well plates were read at 405nm using microplate reader Synergy-HT from BioTek Instruments, Inc (Winnooski, Vermont, USA) and concentration of corticosterones were calculated using 4-parameter logistic curve fitting program software KC4 provided with the equipment (BioTek Instruments Inc., Winnooski, Vermont, USA).

6.2.6. Extraction of carnosine

Carnosine was extracted using a hot water extraction method described by Maikhunthod et al., (2005) with slight modifications. In this method, to one part of meat tissue, 2 parts of pre-cooled (4°C) nano pure water were mixed then homogenized for 3 min (2 times for 1.5min) in a blender (Ostersizer model # 4937, Sunbeam Products Inc., Boca Raton, Florida, USA) (for breast and thigh) and in Polytron® PT2100 homogenizer (Capitol Scientific Inc., Austin, Texas, USA) for brain samples (different homogenizer was used due to small amount of brain tissue as compared to breast and thigh). The

homogenate was centrifuged at 20,000 g for 30 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA); then the supernatant was filtered through Whatman #4 filter paper (GE Healthcare Bio-Sciences Corp., Piscataway, New Jersey, USA). The water extracted filtrate (supernatant) was subjected to a heat treatment at 80°C for 15min in water bath (Precision, Model#283, Thermo scientific, Winchester, VA, USA) then immediately cooled in an ice bath, followed by centrifugation to remove precipitated proteins at 6000 g for 20 min. Supernatant was collected and stored at -80°C until further analysis.

6.2.7. Determination of carnosine by HPLC

Carnosine content in the tissue samples was determined using method described by Gopalakrishanan et al. (1999) and Maikhunthod et al. (2005) with slight modifications. Two ml of tissue sample extract was added to tubes containing 2 ml of 0.4M perchloric acid and vortexed with Vortex Genie 2 ™ (Model# G-560, Fisher Scientific, Bohemia, New York) followed by boiling for 10 min (to precipitate proteins) and then centrifuged at 5000 g for 5 min at 4°C in Sorval RC-5B refrigerated super speed centrifuge (Du-Pont Instruments, Wilimgton, DE, USA). After centrifugation the supernatant was filtered through 0.45µm membrane filter. Filtrate was derivatized using OPA by adding 100 µl of OPA to 500 µl of sample, just prior to injection into HPLC (Shimadzu Instruments, Columbia, MD, USA).

OPA derivatized carnosine was separated using a mobile phase of 0.3M sodium acetate (pH 5.5), methanol and acetonitrile (75:15:10) at the flow rate of 0.75 ml/min on

a Waters Spherisorb SCX-4.6x250 mm column (Waters Corporation, Milford, MA, USA). Derivatized carnosine was detected using a fluorescence detector (RF551 Spectroflurometric detector, Shimadzu Instruments, Columbia, MD, USA) with excitation wavelength of 310nm and emission wavelength of 375 nm. The standard curve was prepared using a carnosine solution (5– 80 mM). The retention time and peak areas were analyzed by EZ start 7.4 Software provided with the equipment (Shimadzu Instruments, Columbia, MD, USA).

6.2.8. Calcium Content

Tissue sample extracts diluted 1:10 before measuring the mineral content. The samples were sent to the Agricultural Services Laboratory at Clemson University where the calcuim content was measured using inductively coupled plasma spectrometer (SPECTRO ARCOS- ICP; Spectro Arcos, Kleve, Germany).

6.2.9. Statistical Analysis

Power analysis was performed to determine the number of birds to use in the study. To perform the power analysis we used preliminary studies to estimate the true mean difference in groups (15) and the standard deviation within the groups (10); and then set the probability of Type I errors at 0.05 and Type II errors at 0.10. The power analysis resulted in sample size of 20 birds to detect a difference of 15mM or greater with standard deviation of 10 and a 0.05 chance of Type I error and 0.10 chance of a Type II

error. SAS (Statistical Analysis Software, Edition 9.2) was used to perform the calculations.

For Statistical analysis of the data, ANOVA was performed, to determine overall differences in the group means while to determine specific differences between pairs of group means, we performed Fisher LSD test. Both tests used a Type I Error rate of 0.05 and SAS (Statistical Analysis Software edition 9.2, SAS Institute Inc., 2007) was used to perform the calculations.

6.3. Results

There was increase in plasma corticosterone levels in stressed compared to non-stressed birds. Corticosterone levels of stressed broilers was 10 fold higher ($p=0.002$) than non-stressed broilers (Table 6.1) which indicated that the birds were stressed. However, there was no difference ($p=0.29$) in heterophil/lymphocyte ratio between non-stressed (0.29) and stressed birds (0.31) (Table 6.1). The increase in corticosterone levels in broilers as compared to heterophil - lymphocyte ratio was explained by Gross and Siegel (1983) who stated that corticosterone levels are indicators of short term stress while heterophil and lymphocytes are the indicators of longer term stress.

Stress increased the carnosine content in breast and thigh of birds, as compared to the non-stress group, while there was no significant change in carnosine content in brain of birds. Carnosine content in breast of stressed birds was 10 times higher ($p=0.005$) than non-stressed birds breast (Table 6.2). Carnosine content in thigh of stressed birds was

approximately 2 times higher (p=0.001) than non-stressed birds. However, carnosine content in the brain of stressed birds did not differ (p=0.82) from non-stressed birds. Thus there was a greater increase in breast and thigh carnosine and corticosterone levels compared to levels of these compounds in the brain in response to short term stress (Figure 6.1).

6.4. Discussion

There was no significant difference (p=0.29) in heterophil/lymphocyte ratio and this lack of difference in heterophil/lymphocyte ratio of non-stress birds (0.29) and stress birds (0.31) confirms previous work (Altan et al., 2000; Bedanova et al., 2007; Siegel and Gross, 2000). These researchers found that the reason for a lack of change in the heterophil lymphocyte ratio in reponse to short-term stress was the less time for physiological change in these cells. Zulkifli (2000) and Gross (1990) indicated that any change in heterophil lymphocyte ratio in response to stress would require at least 20 hours or more after stress.

Corticosterone levels for non-stressed chickens in the present study were slightly higher (2275.46 pg/ml) than those reported by other researchers (Bedanova et al., 2007) who studied stress in broilers at different time intervals during shackling. The reason for the higher levels of corticosterones in the present study might be due to crating, ambient disturbances as well unfamiliar personnel handlers for the broilers, which could induce stress even among the non-stressed group of chickens (Knowles and Broom, 1990). The

corticosterone levels in stressed chickens in the present experiment were similar to corticosterone levels as measured by these same researchers.

Crating also causes increase in the corticosterone levels which could be a potent stressor (Kannan and Mench, 1996). Moreover, it was concluded by Kannan and Mench that upright handling is less stressful to the broilers than inverted handling (Kannan and Mench, 1996; Kannan and Mench, 1997). However, rough inverted handling is practiced in commercial facilities which augment the physiological stress among chickens (Kannan and Mench, 1996; Kannan and Mench, 1997) . Increased corticosterone levels in stressed compared to non stress broilers were in accordance with previous work (Bedanova et al., 2007; Kannan and Mench, 1996; Kannan and Mench, 1997) and supports the conclusion drawn by Gross and Siegel (1983) that corticosterone levels are the indicators of short time stress while heterophil and lymphocytes are the indicators of long time stress.

6.4.1. Discussion of carnosine levels

Breast tissues are more susceptible to stress than thigh tissue (Lin et al., 2006). In present study, the levels of carnosine found in breast and thigh muscles were higher than the levels determined by Maitkhthund and Intarapichet (2005). Maikhunthod and Intarapichet (2005) reported carnosine levels of 2900.1 μg/gm for chicken breast muscle and 419.9 μg/gm for thigh, about 7 times higher in breast compared to thigh muscle. These researchers extracted carnosine at 60, 80, 100°C and with ultrafiltration (500 MW cut off). We used hot water extraction (80°C for 15 min) instead of 80°C for 10 min or

100°C for 10 min used by these authors, our treatment might improve the recovery of carnosine from these tissues. Therefore, it is possible that this discrepancy arises due to methodological differences, sample collection site and sample treatment differences in birds. We found that with parallel increase in carnosine and corticosterone levels and these results agree with previous authors who injected carnosine in the brain of chicks and found increase in the levels of corticosterones (Tomonaga et al., 2004; Tsuneyoshi et al., 2007).

Very little information is available on the effect of stress on carnosine levels and the following discussion attempts to explain the reasons and mechanism behind carnosine elevation during stress.

6.4.2. Carnosine increase might be due to protein degradation

During stress, protein synthesis decreases while protein catabolism rate increases (Dong et al., 2007). It is likely that carnosine level is a by-product of protein hydrolysis and certain enzymes are activated resulting in breakdown of proteins. Calpain I and calpain II or (μ calpain or m calpain) are Ca-dependent proteases present in animal cells and belong to the calpain family. In normal skeletal muscle, calpain and calpastatin are located on or next to Z-disk with smaller amount on I-band (Goll et al., 2003). The activity of μ/m calpain system is greater in breast and thigh (approximately 92%) than in brain (72.9%) (Lee et al., 2007). During stress, corticoterone levels increase, accompanied by proteolysis, which could be due to calpain system. Corticosterones are known to increase muscle calpain activity (Hayashi et al., 2000), and we found that there

was an increase in calcium levels in muscles with stress (Table 6.2). There is a relationship between calcium concentration with activation of μ or m calpain (Lee et al, 2007), and it is likely that μ calpain may be activated during stress. It was also reported that μ calpain activity is greater in breast (8.9%) and thigh (6.6%) than in brain (1.1%) (Lee et al., 2007) which may explain the greater change in carnosine levels in breast and thigh compared to brain.

6.4.2. Carnosine relationship with creatine kinase

Corticosterone administration to chickens increases oxidative stress on cells accompanied by increasing proteolysis and gluconeogensis (Gao et al., 2008) and in increased plasma uric acid, and creatine kinase activity (Lin et al., 2004a and 2004b). There is a possible relationship between creatine kinase and carnosine. Kannan et al. (2000) reported that plasma creatine kinase activity peaked during transportation, and that creatine kinase increased with corticosterone levels up to 2 hr after which these compounds decreased. A study on exercised horses indicated that there is a direct relationship between carnosine and creatine kinase during stress, that is, with an increase in creatine kinase there is an increase in carnosine (Dunnett and Harris, 2002). In the same study, they found an increase in plasma carnosine concentration after exercise from 5 to 30 min followed by a decrease in carnosine concentration at 120 min and this concentration returned to normal level after one day (concentration after 24 hr was equal to pre-exercise). In comparison, during the present study with chickens, birds were stressed for 15 min and it is possible that at this time carnosine was at peak levels due to

stress in skeletal muscles which may decrease over time interval, as seen in exercised horses. Therefore, more studies needed to be conducted at different time intervals to find the exact correlation of carnosine with stress.

6.4.4. Formation of histamine from carnosine during stress

Flancbaum et al. (1990) suggested that carnosine plays crtitical role in histidine-histamine metabolic pathways during stress. Carnosine acts as a reservoir for histidine (Flancbaum et al., 1990a) which converts to histamine when demand arises during stress or injury (Flancbaum et al., 1990b).

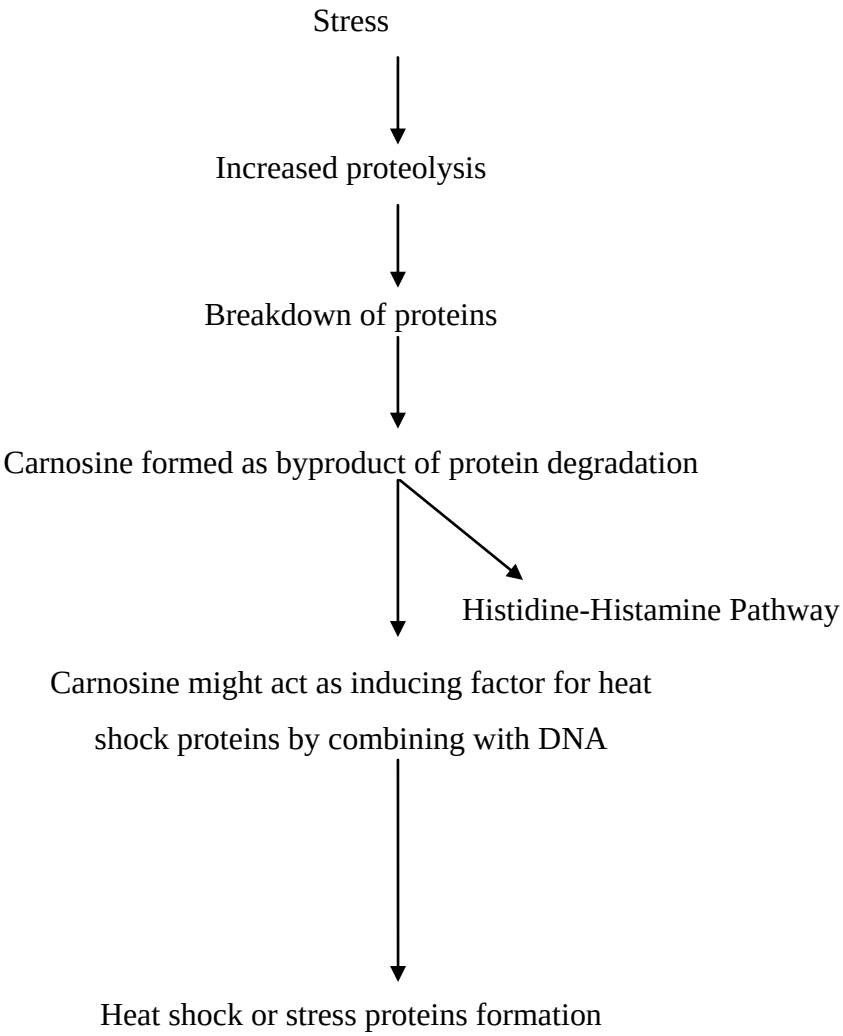
6.4.5. Relationship to Heat Shock proteins

During stress aggregated or misfolded proteins creates a large pool of non protein substrates that compete with heat shock factors and carnosine may be one of them. Released heat shock factor assembles to DNA trimmers and results in elevated transcription, synthesis and accumulation of stress proteins. Known heat shock transcription factors include salicylates, prostaglandins, arachidonic acid and amino acid analogs which play roles in inflammatory responses (Morimoto, 1993).

It has been reported that zinc-carnosine induces HSP72 (heat shock protein72) in gastric mucosa of rats (Odashima et al., 2002). NF-κB is a ubiquitous transcripition factor which regulates transcription of many proinflammatory genes such as interleukin (IL)-1β, IL-8 and tumor necrosis factor (TNF)-α. The same authors reported that along with the induction of HSP72 protein, zinc-carnosine also suppresses NF-κB (Nuclear

Factor- κB) activation and thus prevents mucosal injury and they suggested that zinc-carnosine could be a potent chaperon (Odashima et al., 2006). Similar conclusions were drawn by Mikami et al (2006), in addition they also found that along with zinc-carnosine, L-carnosine can also induce HSP72 (Mikami et al., 2006). Therefore, this indicates that carnosine might facilitate expression of heat stress genes and induction of stress shock proteins.

Figure 6.2: Suggested possible Pathway



6.5. Conclusions

The present study indicates that carnosine plays a significant role during stress. Increase in carnosine levels in response to stress could be temporary and may reduce after a certain time as the corticosterone levels return to normal approximately 3 hr after initial stress. Additional research should be conducted to measure the carnosine levels at different time intervals of stress in conjunction with corticosterone, creatine kinase, carnosine synthetase activity, carnosinase activity, calpain activity etc. to better understand and evaluate carnosine's role during stress. There are few published reports on the relationship of carnosine to stress. This study found a relationship between carnosine, corticosterone and short term stress which was different between muscle and brain tissues.

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TABLES and FIGURES

Table 6.1: Corticosterone levels and heterophil-lymphocyte ratio of stress and non stress broilers

Treatment	Heterophil (# of cells)	p-values	Lymphocyte (# of cells)	p-values	Heterophil Lymphocyte Ratio	p-values	Corticosterone Assay (pg/ml)	
Non-stress	22.14±1.33 [a]	P=0.697	76.43±0.64[a]	P=0.661	0.29±0.018[a]	P=0.59	2275.46±0.11[a]	P=0.002
Stress	23.11±1.69[a]		75.77±1.42[a]		0.31±0.03[a]		24358.67±1.84[b]	

1. Mean± S.E.M;
2. Total number of slides counted for heterophil- lymphocyte ratio was 10 and total # of cells counted per slide was 100.
3. For corticosterone assay (N=3)
4. Fisher's Least Significant Difference Test was used to compare mean values; [a-b] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).

Table 6.2: Carnosine levels in different tissues of stress and non-stress broilers.

Organ	Treatment	Calcium content ppm	p-value	Carnosine Content (wb)[2, 3]	p-value	Carnosine Content (db)	p-value
Breast	**Non-stress**	6.74±0.07 [a]	P=0.001	1.85±0.24 [1, a]	P=0.005	7.52±0.97[a]	P=0.0005
	Stress	11.03±0.13 [b]		17.39±1.33[b]		70.89±5.39[b]	
Thigh	**Non-stress**	10.50±0.37 [a]	P=0.006	11.10±1.02[a]	P=0.001	44.47±4.08[a]	P=0.002
	Stress	13.99±0.19 [b]		21.25±1.25[b]		85.12±5.01[b]	
Brain	**Non-stress**	8.48±0.38 [a]	P=0.002	10.16±1.53[a]	P=0.82	45.44±12.37[a]	P=0.54
	Stress	13.78±0.24 [b]		10.27±2.77[a]		55.12±14.88[a]	

1. All values are in Mean± S.E.M (N=5)
2. wb= wet basis; db= dry basis
3. Carnosine content is expressed in mg/gm of the original sample.
4. Fisher's Least Significant Difference Test was used to compare mean values; [a-b] similar letters indicate that the means values are not significantly different ($p \geq 0.05$); while different letters indicate that the mean values are significantly different ($p \leq 0.05$).

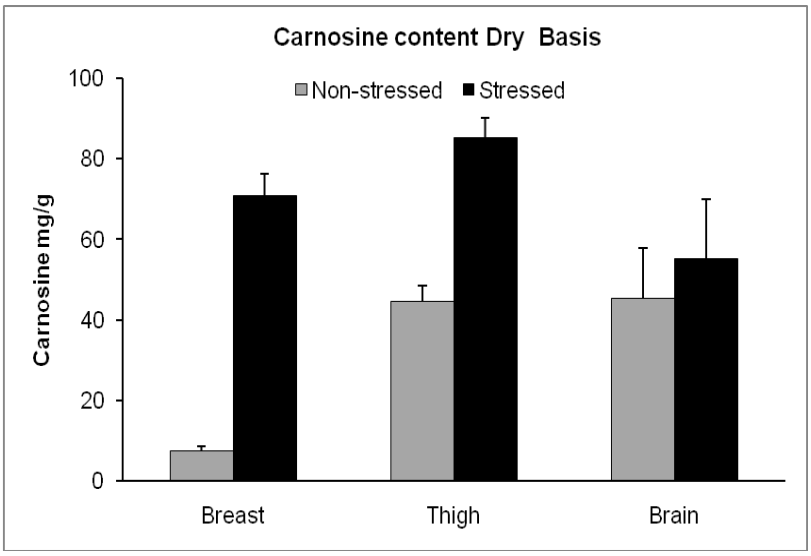

Figure 6.1: Change in carnosine levels in different tissues during stress. Breast and thigh carnosine levels were significantly different ($p \leq 0.05$). Brain carnosine levels were not significantly different ($p \geq 0.05$).

APPENDIX

Table 1A: (Stage 1) Extraction of Carnosine from organ samples (Mass Balance)

Organ	Sample wt.	Water Wt.	Other/Loss	Final Extract
Tail	52.47±2.54	105±5	76.37±3.23	68.64±1.80
Gizzard	53.04±3.53	97.73±13.55	68.12±10.10	85.44±3.28
Liver	67.19±6.66	117.76±4.54	69.24±6.14	93.48±11.76
Head	55.12±5	110±10	79.82±9.79	85.04±1.87
Brain	62.64±2.20	113.52±7.93	68.13±0.77	90.26±12.71
Lungs	60.64±0.35	120.19±0.19	47.63±4.9	124.69±6.59
Heart	59.95±0.25	120±0	48.68±2.03	118.31±4.06

Mean± SEM (n =3)

All weights are in grams

Table 2A: (Stage2) Ultrafilteration to purify extract (Mass Balance)

Organ	Extract wt.	Other/Loss	Final Extract
Tail	40.17±9.79	5.94±2.23	34.22±7.59
Gizzard	27.76±0.33	1.26±0.175	26.49±0.50
Liver	30.94±1.11	1.08±0.51	29.85±0.62
Head	16.2±1.2	5.37±0.37	11.09±0.54
Brain	83.48±3.18	44.18±4.38	38.63±1.40
Lungs	30.02±0.42	4.24±0.89	25.77±1.81
Heart	29.56±0.33	2.52±0.43	27.04±0.13

Mean +SEM (n=3)

All weights are in grams

Table 3A: (Stage3) Freeze drying (Mass Balance)

Organ	Ultrafiltrate initial wt	Dry ultrafiltrate wt	% Moisture	% Dry solids
Tail	13.69±1.22	0.12±0.04	99.17±0.18	0.83±0.18
Gizzard	13.5±2.89	0.11±0.03	99.24±0.09	0.75±0.09
Liver	14.52±0.48	0.31±0.02	97.89±0.18	2.11±0.18
Head	19.46±0.05	0.11±0.01	99.45±0.08	0.54±0.08
Brain	15.01±0.26	0.12±0.02	99.42±0.09	0.76±0.09
Lungs	16.05±3.09	0.11±0.01	99.3±0.23	0.7±0.23
Heart	16.22±0.26	0.12±0	99.26±0.01	0.74±0.01

Mean ±SEM (n=3)

All weights are in grams

Figure 1A: HPLC chromatogram of tissue ultrafiltrate- gizzard

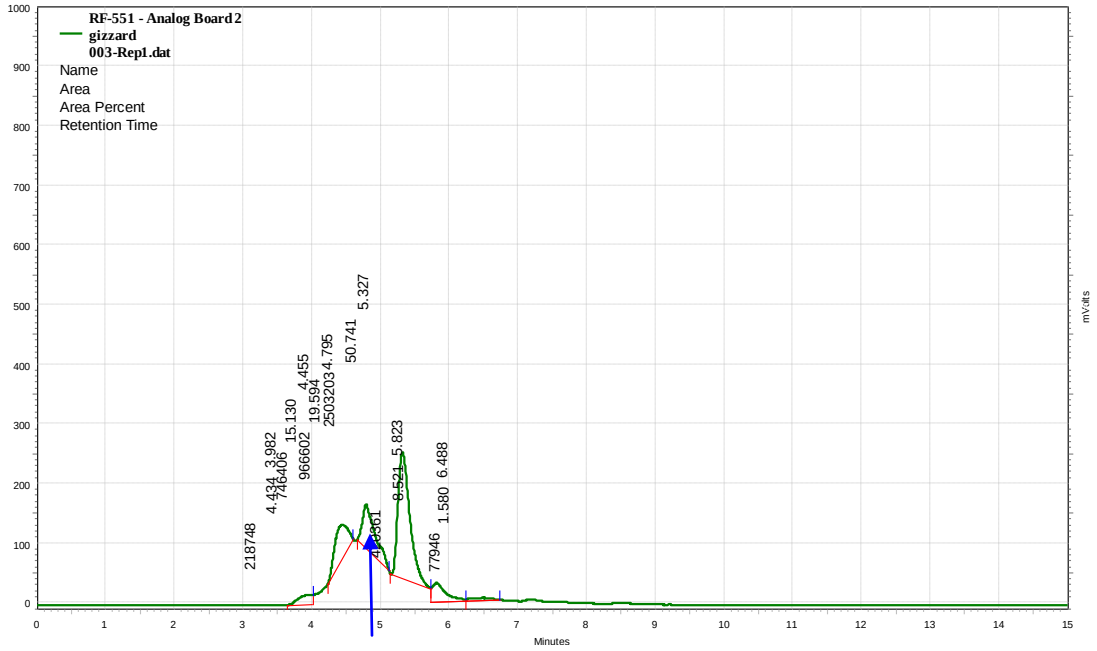

Figure 2A: HPLC chromatogram of tissue ultrafiltrate- heart

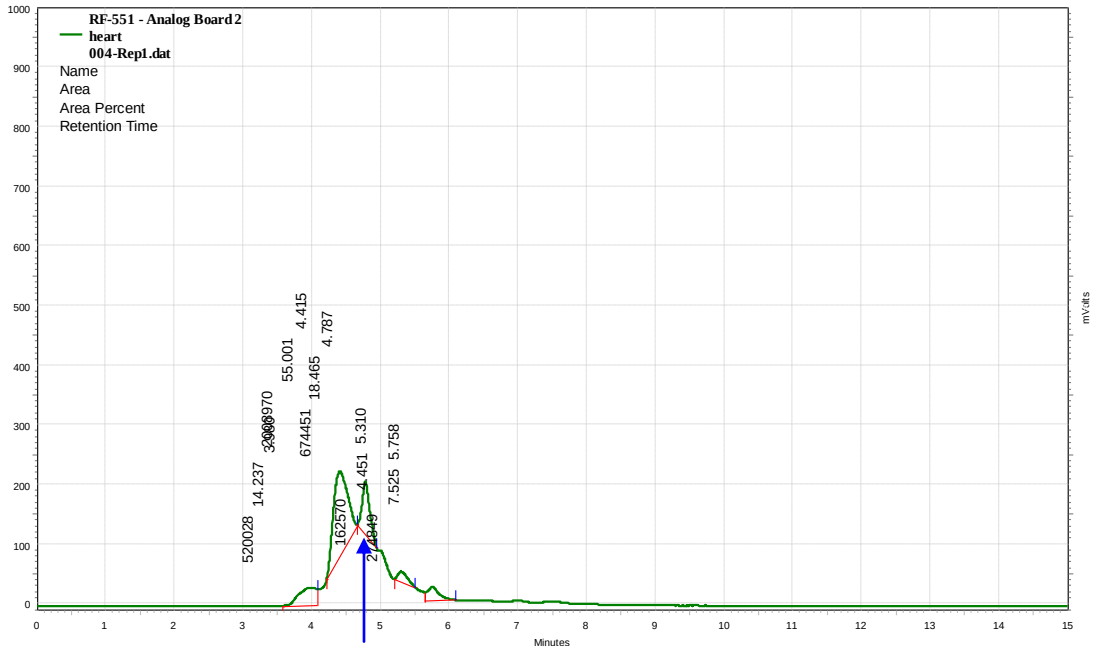

Figure 3A: HPLC chromatogram of tissue ultrafiltrate- tail

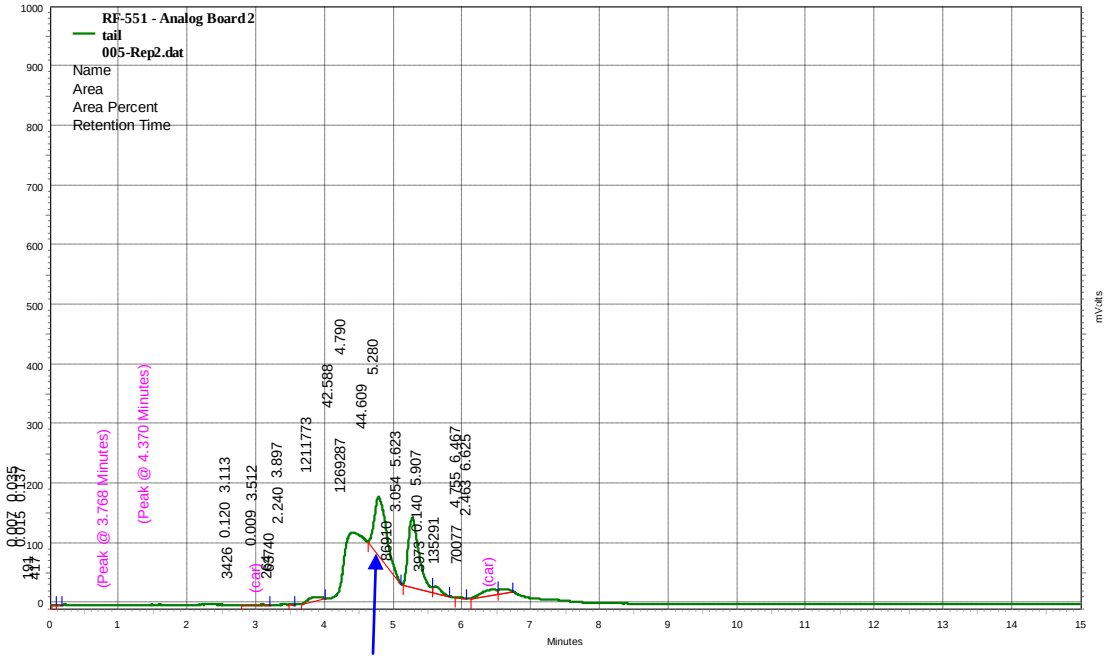

Figure 4A: HPLC chromatogram of tissue ultrafiltrate- head

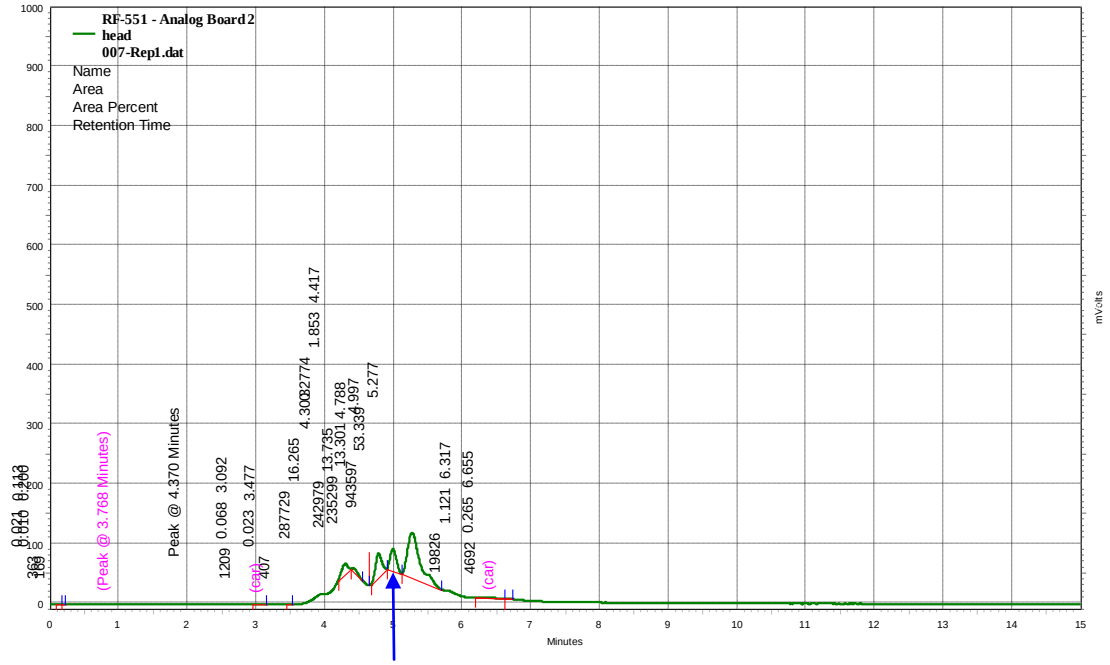

Figure 5A: HPLC chromatogram of tissue ultrafiltrate- brain

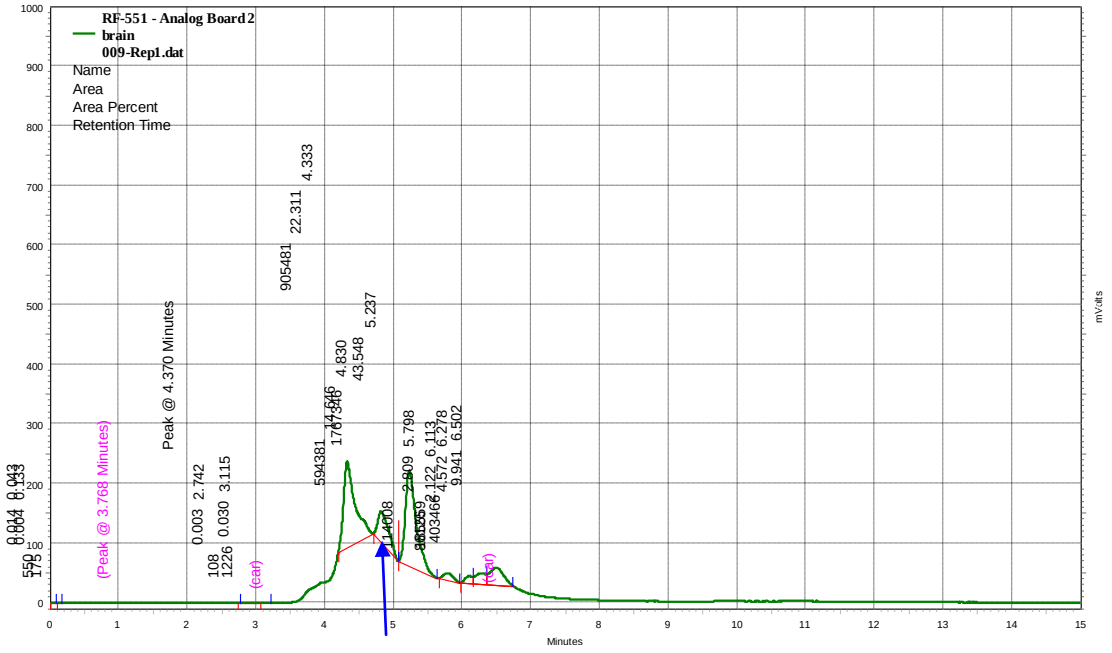

Figure 6A: HPLC chromatogram of tissue ultrafiltrate- liver-1:15 dilutions

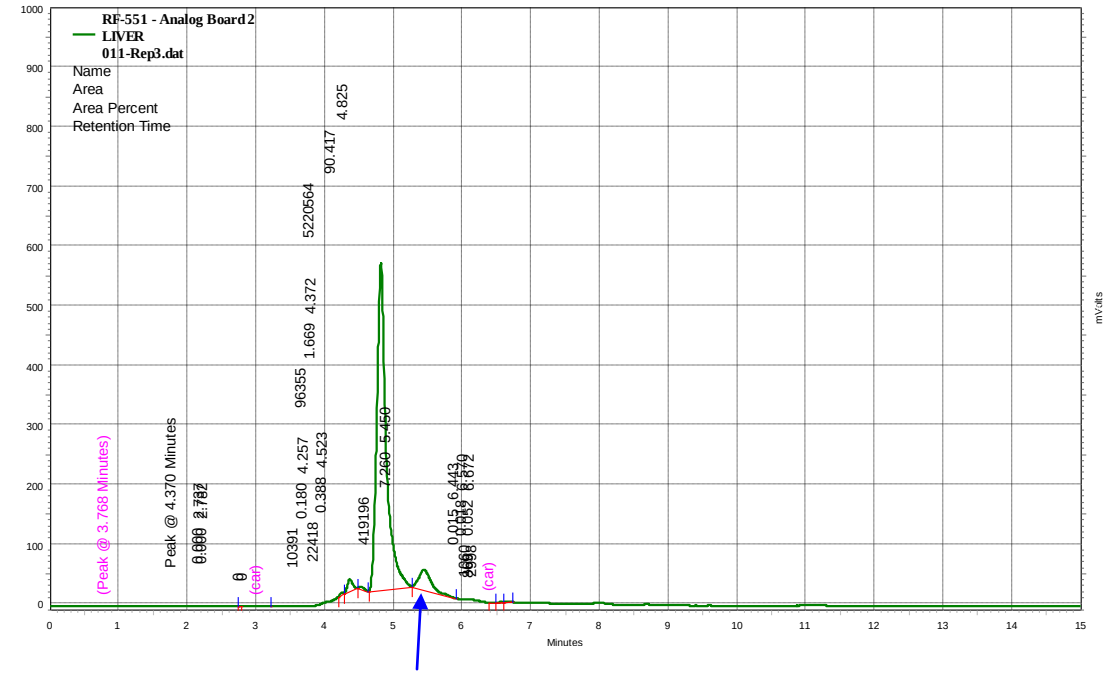

Figure 7A: HPLC chromatogram of tissue ultrafiltrate- lungs

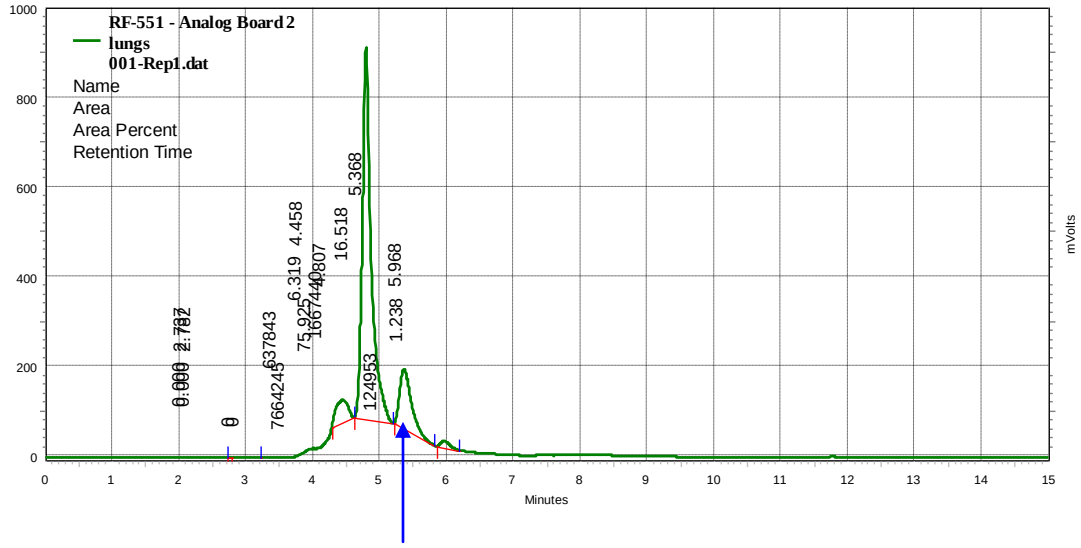

Figure 8A: HPLC chromatogram of organ ultrafiltrate- liver-1:15 dilutions spiked with histidine, anserine and carnosine (1:1:1 ratio)

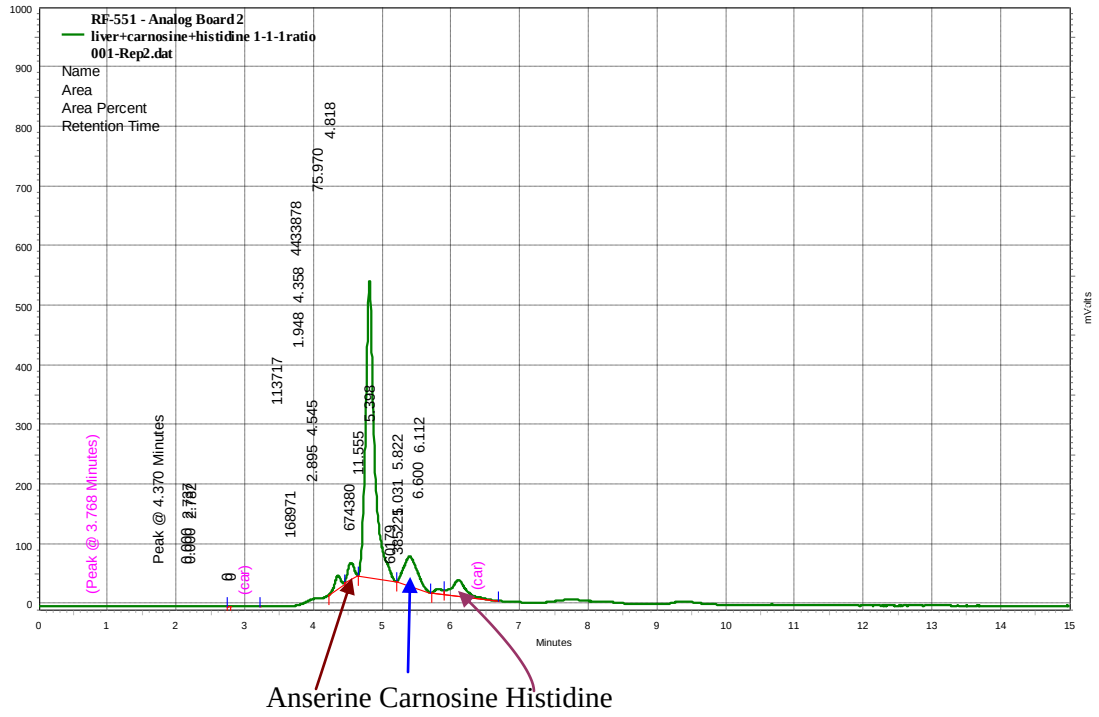

Figure 9A: HPLC chromatogram of reconstituted gizzard dry powder (25mg/ml)

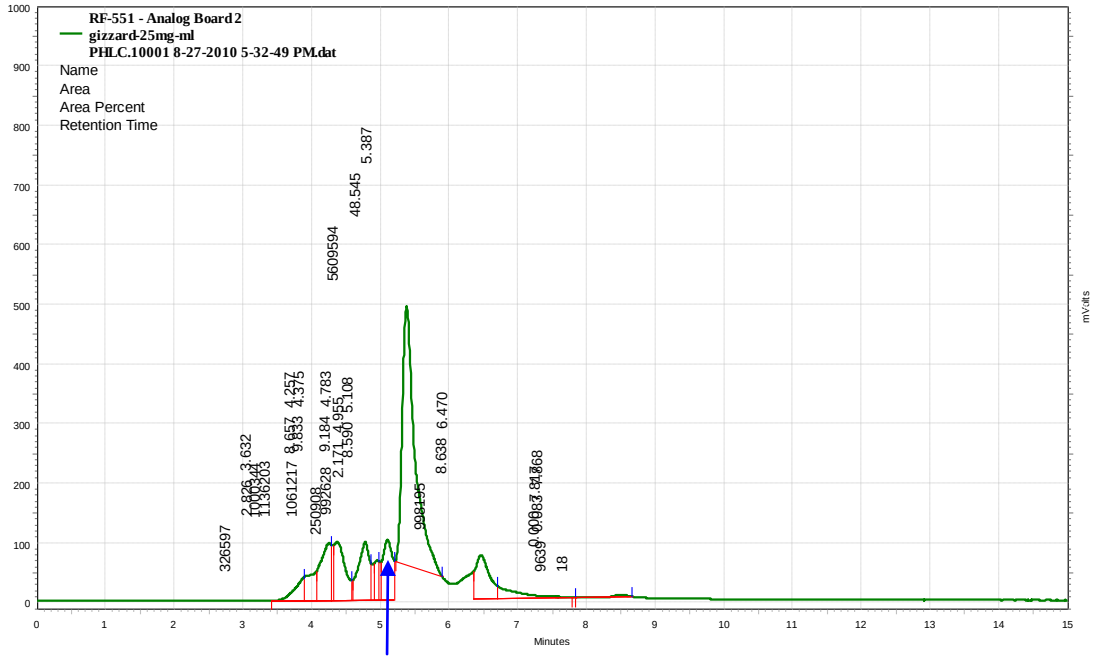

Figure 10A: HPLC chromatogram of reconstituted heart dry powder (25mg/ml)

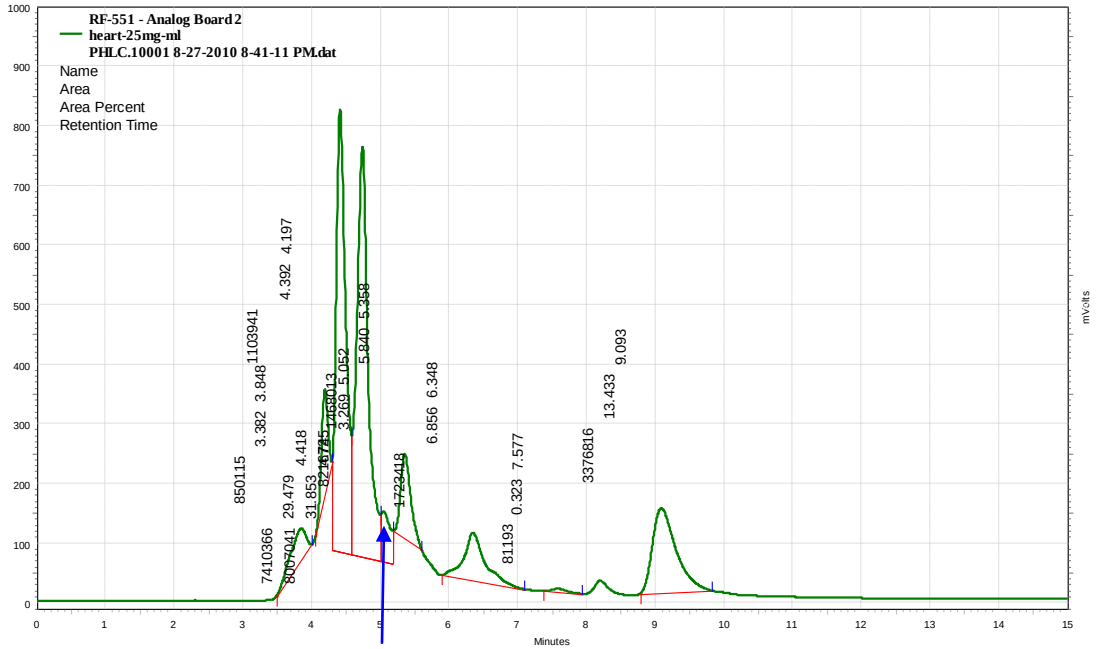

Figure 11A: HPLC chromatogram of reconstituted brain dry powder (25mg/ml)

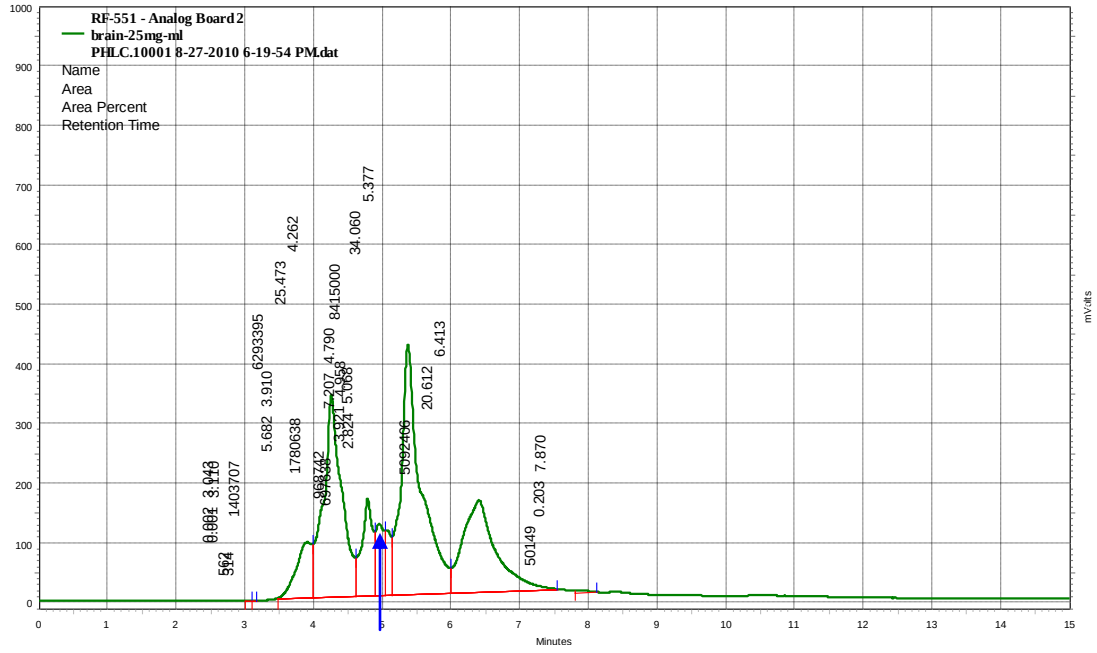

Figure 12A: HPLC chromatogram of reconstituted liver dry powder (25mg/ml)

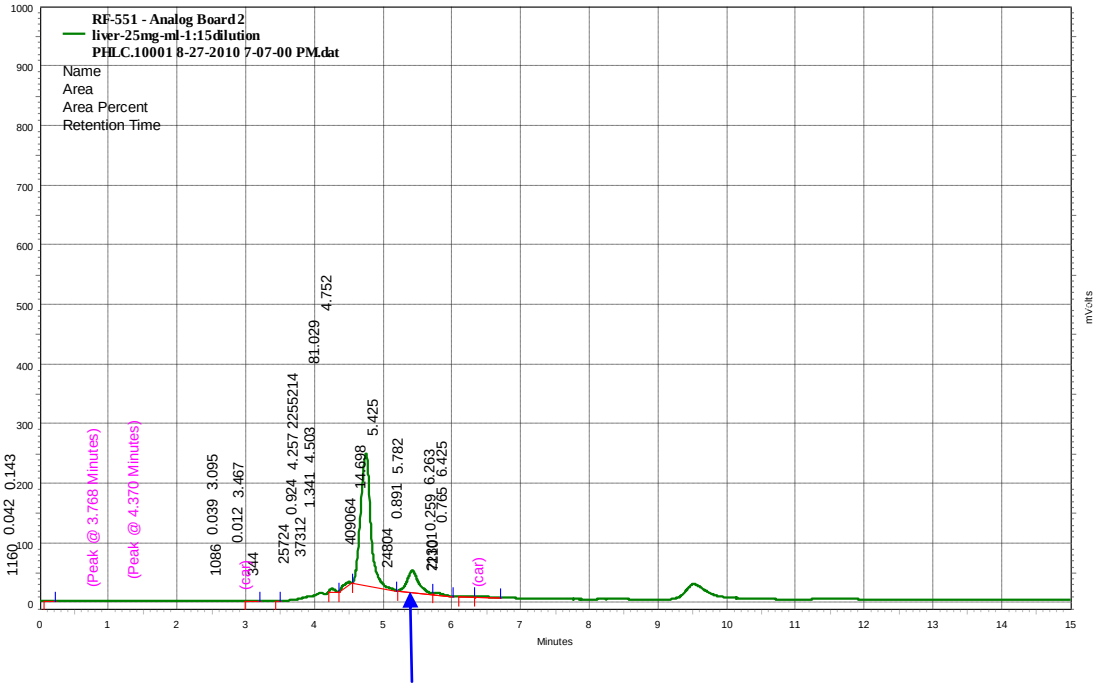

Figure 13A: HPLC chromatogram of reconstituted tail dry powder (25mg/ml)

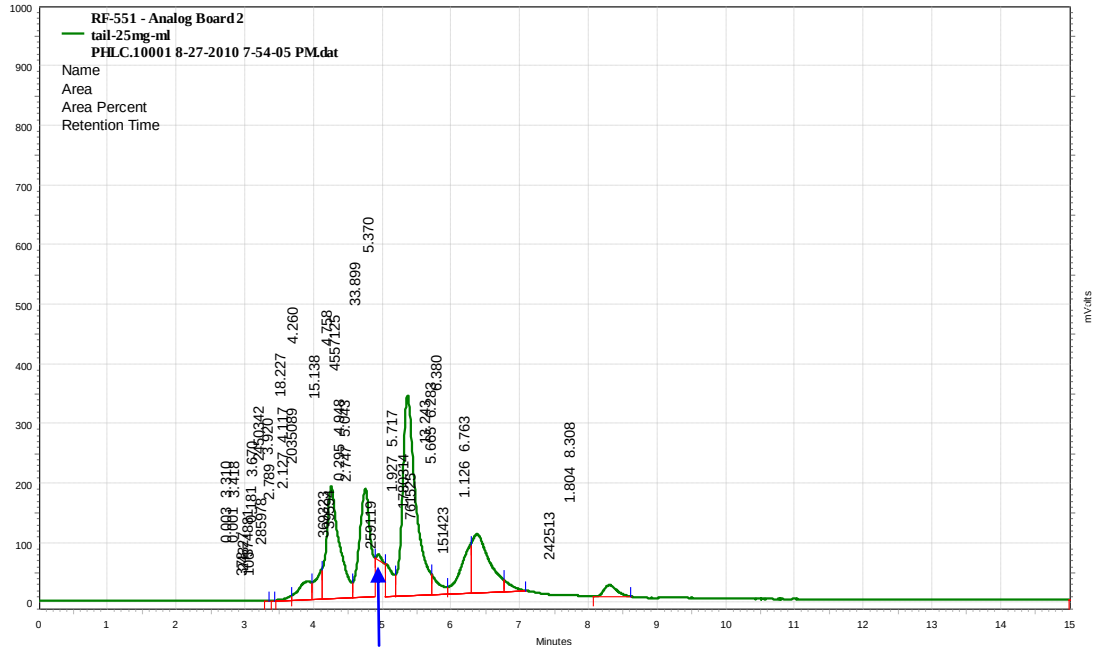

Figure 14A: HPLC chromatogram of reconstituted lungs dry powder (25mg/ml)

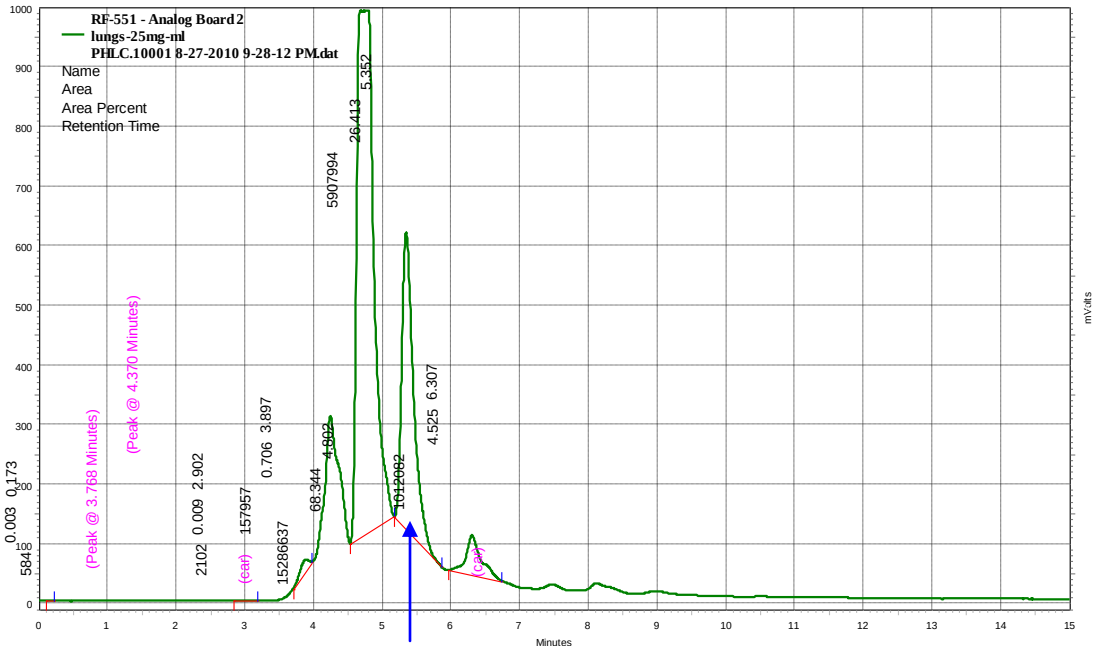

Figure15A: HPLC chromatogram of reconstituted head dry powder (25mg/ml)

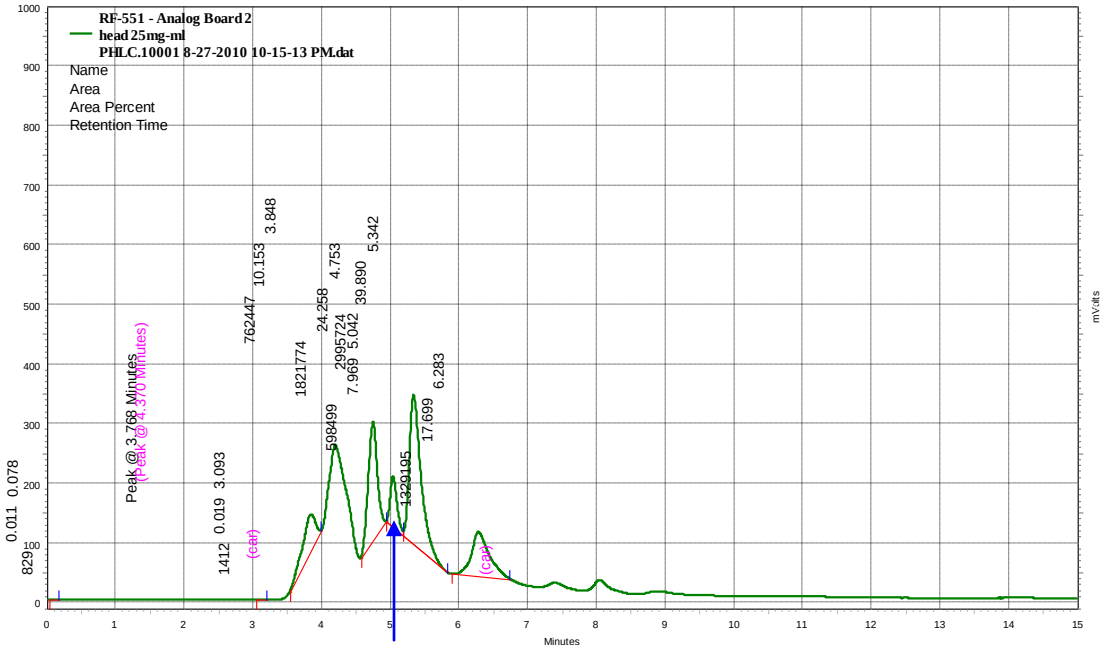

Figure 16A: HPLC chromatogram of Poultry protein meal- sample-A

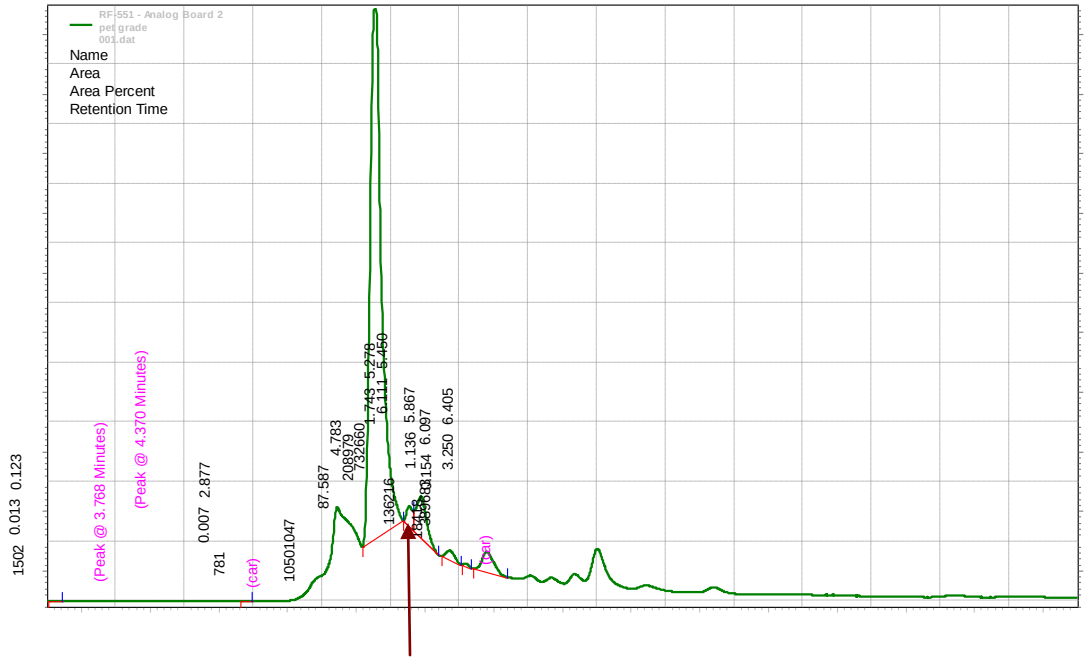

Figure 17A: HPLC chromatogram of Poultry protein meal- sample-A spike

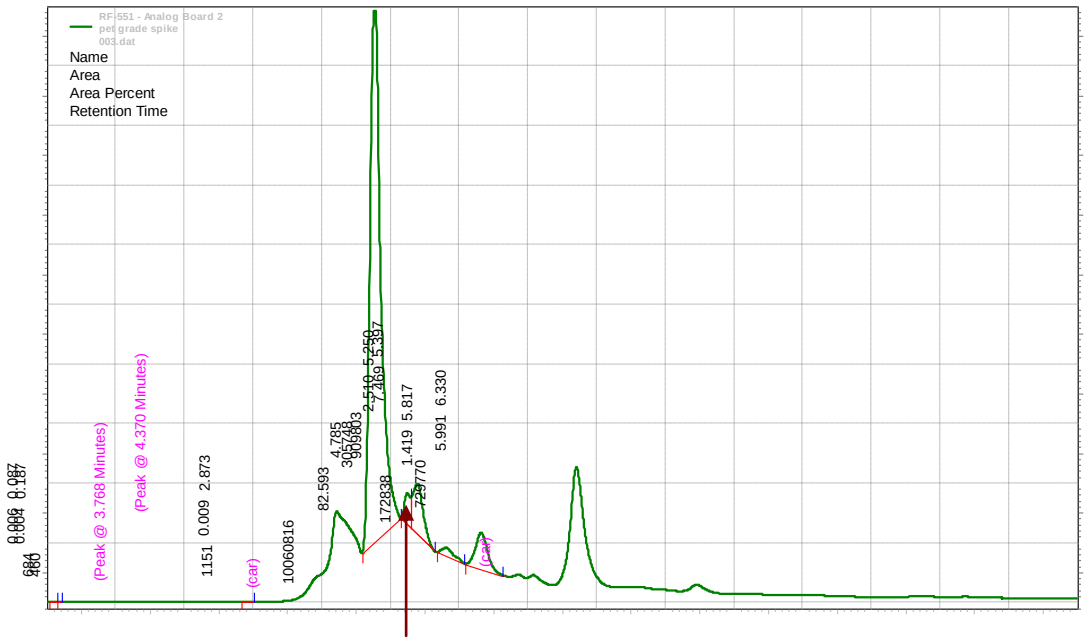

Figure 18A: HPLC chromatogram of Poultry protein meal- sample-G

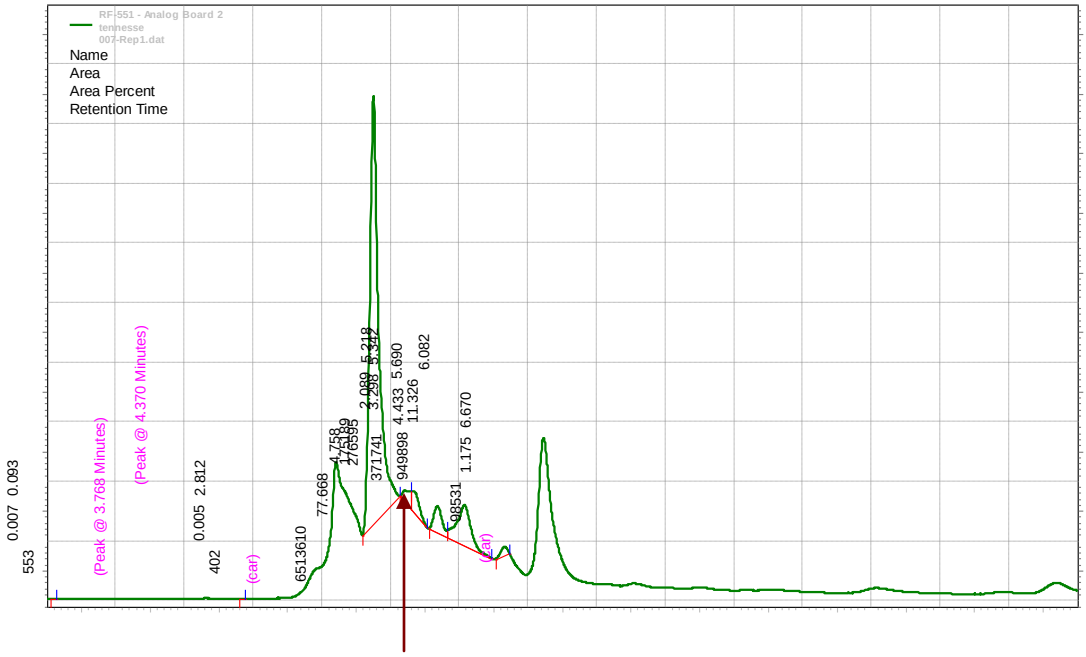

Figure 19A: HPLC chromatogram of Poultry protein meal- sample-G- spike

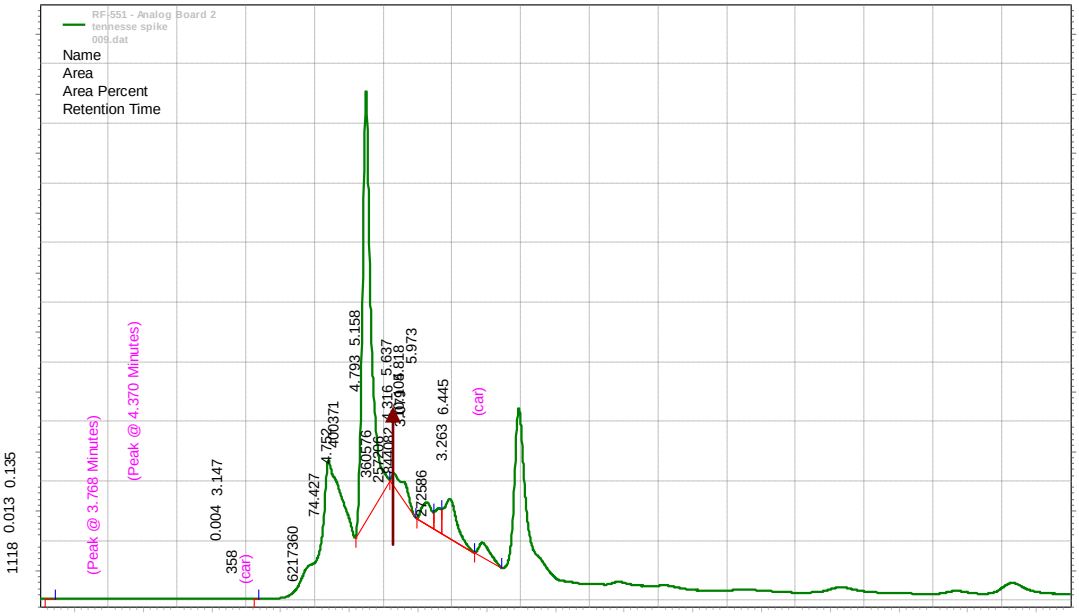

Figure 20A: HPLC chromatogram of carnosine standards at different concentrations.

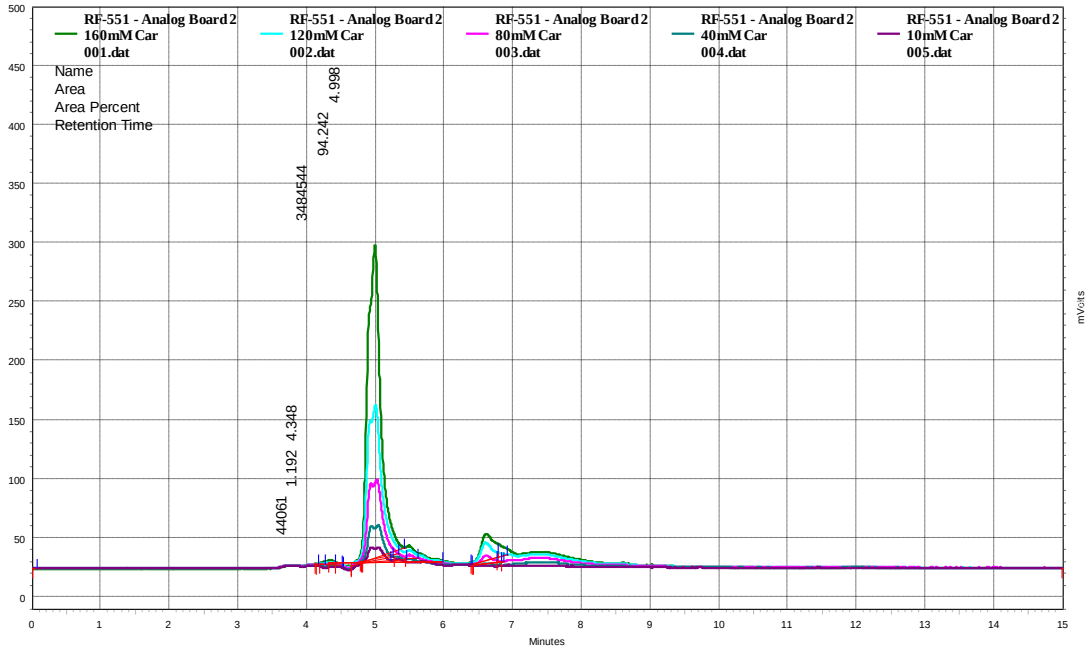

Figure 21A: HPLC chromatogram of breast-non stress sample

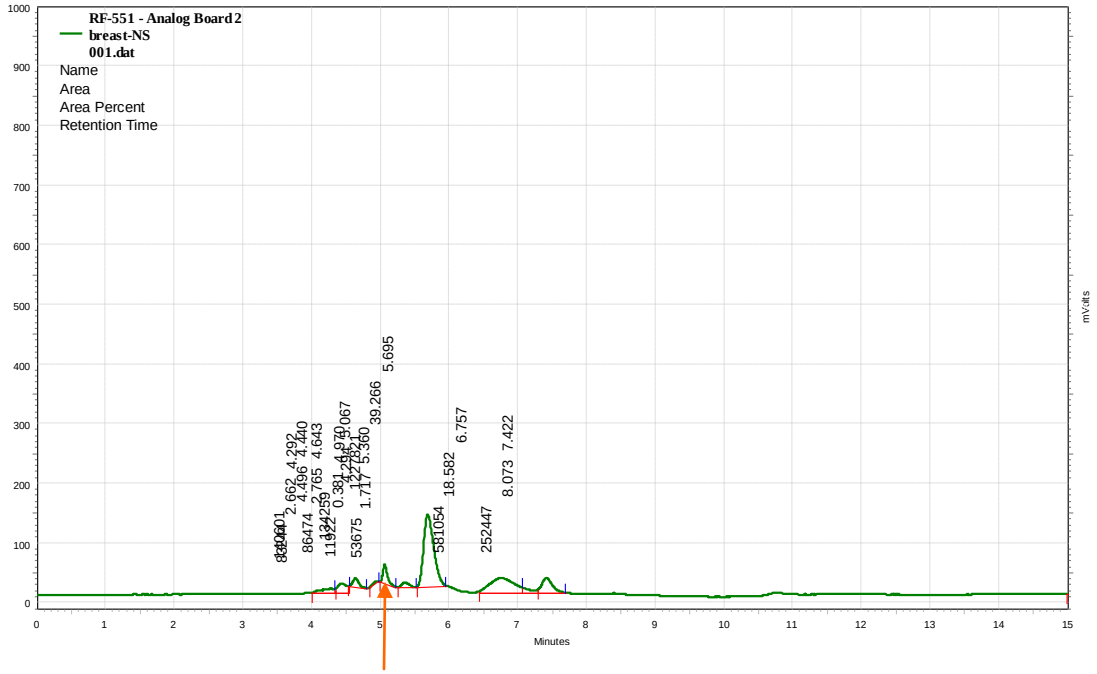

Figure 22A: HPLC chromatogram of breast-stress sample

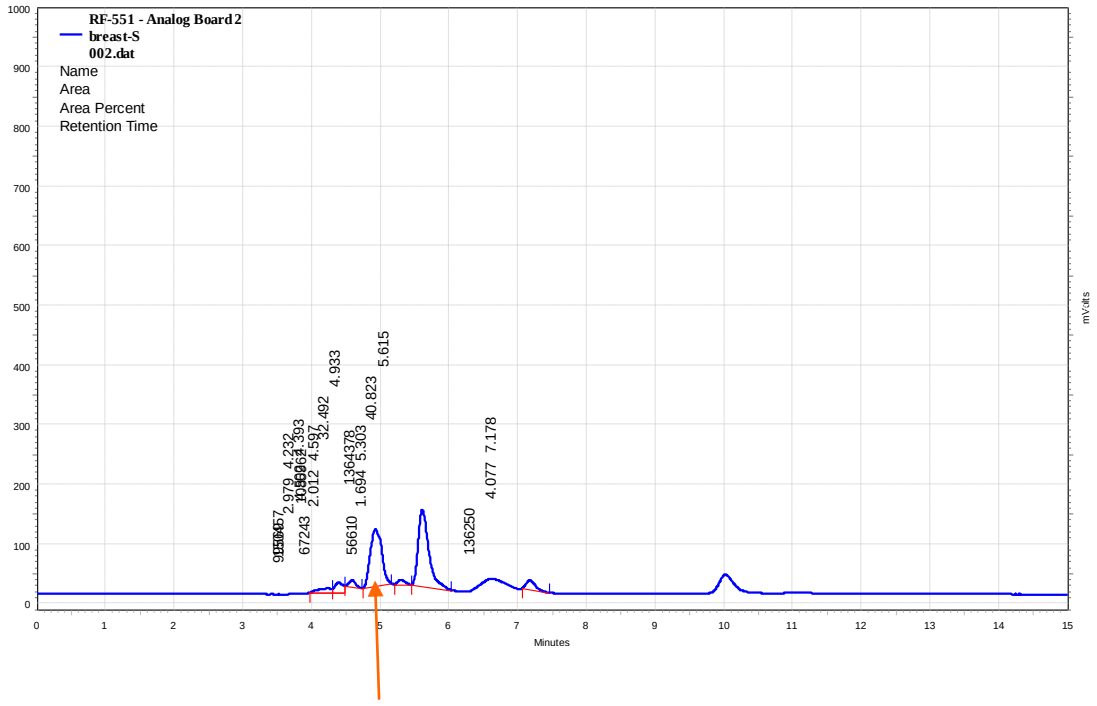

Figure 23A: HPLC chromatogram of thigh-non stress sample

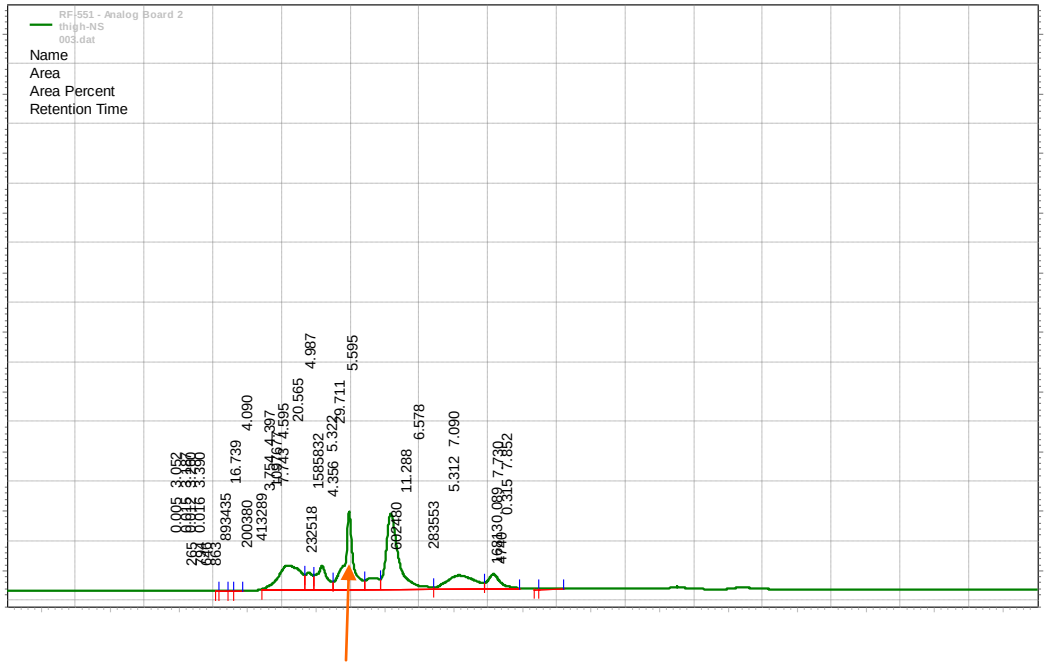

Figure 24A: HPLC chromatogram of thigh-stress sample

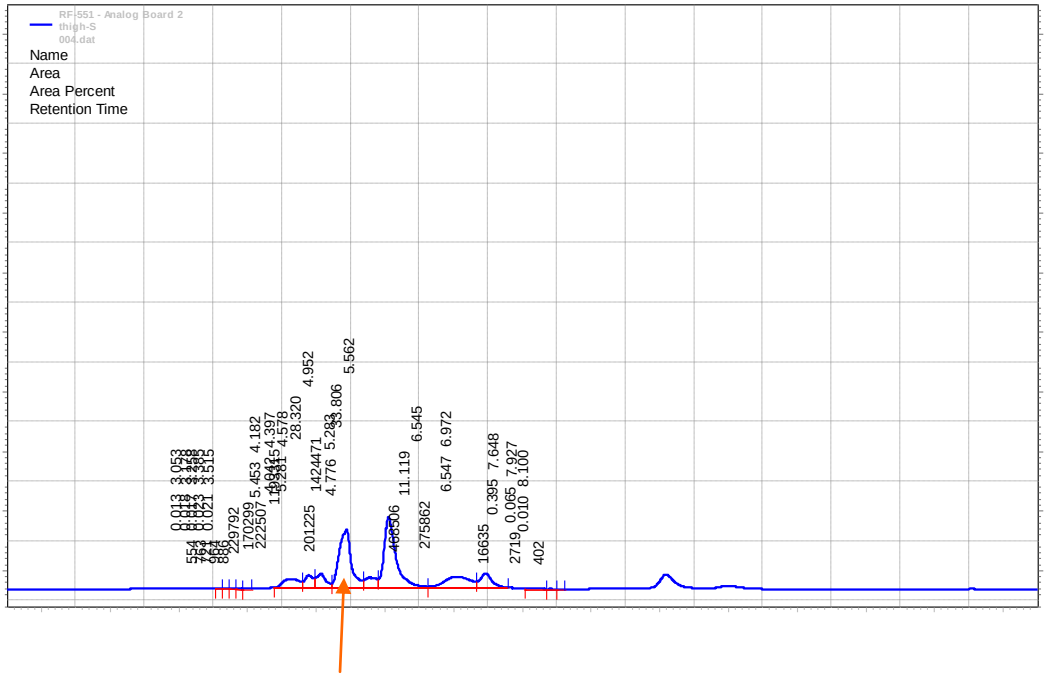

Figure 25A: HPLC chromatogram of brain-non stress sample

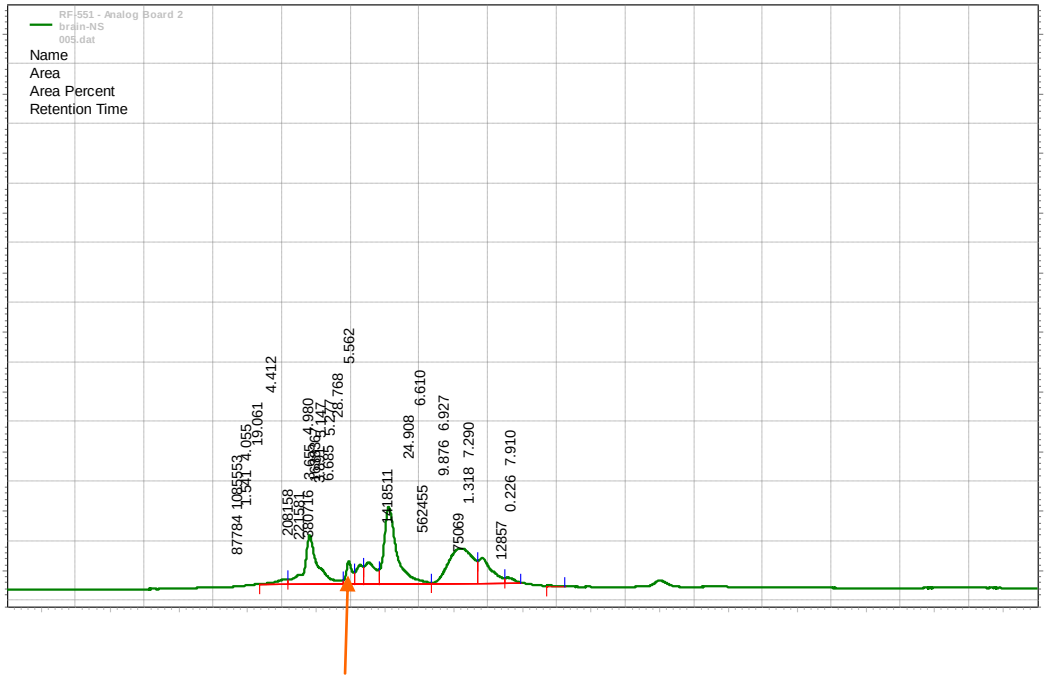

Figure 26A: HPLC chromatogram of brain-stress sample